

GENETIC REQUIREMENTS IN T CELL-MEDIATED IMMUNE RESPONSES  
TO VIRUS INFECTION

by

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There could be no fairer destiny for  
any ..... theory than that it should  
point the way to a more comprehensive  
theory in which it lives on, as a  
limiting case.

Albert Einstein



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## A C K N O W L E D G E M E N T S

The work described in this thesis was performed during the tenure of an Australian National University Ph.D Scholarship, for which I am grateful.

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H-Y male specific antigen

Ig immunoglobulin

i.p. intraperitoneal

i.v. intravenous

I<sub>A</sub> I region associated

I<sub>E</sub> immune response gene

3T3 murine fibroblast cells (H-3<sup>+</sup>)

LCM lymphocytic choriomeningitis

Ly lymphoid tissue antigen loci

2ME 2-mercaptoethanol

MEM Eagle's minimal essential medium

MHC major histocompatibility complex

MIR mixed lymphocyte reaction

## A B B R E V I A T I O N S   U S E D

B cell	Bursa of Fabricius (or mammalian equivalent)-derived lymphocyte
CMI	cell-mediated immunity
CML	cell-mediated lympholysis
DTH	delayed-type hypersensitivity
EID	egg infectivity dose
F9	murine teratocarcinoma cells
F-15	Eagle's minimal essential medium with nonessential amino acids
FCS	foetal calf serum
FLV	Friend leukemia virus
GVHR	graft-versus-host reaction
H	histocompatibility
HLA	major histocompatibility complex in man
HSV	herpes simplex virus
H-Y	male specific antigen
Ig	immunoglobulin
i.p.	intraperitoneal
i.v.	intravenous
Ia	I region associated
Ir	immune response gene
L929	murine fibroblast cells (H-2 <sup>k</sup> )
LCM	lymphocytic choriomeningitis
Ly	lymphoid tissue alloantigen loci
2ME	2-mercaptoethanol
MEM	Eagle's minimal essential medium
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction



MOI	multiplicity of infection
MSV	murine sarcoma virus
NIAID	National Institute of Allergy and Infectious Diseases
P815	murine mastocytoma cells (H-2 <sup>d</sup> )
PBS	phosphate-buffered saline
PFU	plaque-forming unit
S.E.	standard error of the mean
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
SV40	simian virus 40
T cell	thymus-derived lymphocyte
T <sub>C</sub>	cytotoxic T cell
TL	thymus leukemia antigen
Thy.1	theta (θ) antigen
TNBS	trinitrobenzenesulphonic acid
TNP	trinitrophenyl
V genes	variable genes
VEE	Venezuelan equine encephalitis

## A I M S   A N D   S U M M A R Y

Cytotoxic thymus-derived lymphocytes (T cells) are a prominent part of the immune response to several groups of viruses. In the case of ectromelia virus infection in mice which causes mousepox they seem to be essential elements in the process of recovery from primary infection. An intriguing aspect of these T cells is that they apparently recognize both virus-specific and host-specific components on virus-infected cells. In mice, the host-specific components seem to be coded by gene(s) in either the *H-2K* or *H-2D* region of the major histocompatibility complex (MHC). Experiments described in this thesis concern various aspects of the phenomenon of H-2 restricted responses to ectromelia virus infection. The objectives were to assess the antiviral potential of effector T cells *in vivo* on the one hand and the further elucidation of the role of the MHC in cellular interactions on the other hand.

Cell transfer experiments using mice with recombinant H-2 haplotypes were used to map the *H-2* regions which must be shared by ectromelia-immune T cell donors and virus-infected recipients for transfer of virus clearance mechanisms with spleen cells. These experiments revealed that the same rules for H-2 restriction, as observed with cytotoxic T ( $T_C$ ) cells *in vitro*, applies to the transfer of antiviral activity *in vivo*. Sharing of *K* or *D* region genes between donors and recipients of virus-immune T cells was necessary and sufficient; *I* region genes were not involved. Investigations with secondary ectromelia effector cells generated *in vitro* indicated that depending upon the order of administration of virus-immune T cells and virus, cells from cultures are capable of conferring antiviral activity and this could be of clinical therapeutic value in the future.

Since *in vitro* systems offer far more control over experimental



variables than *in vivo* systems, methods for the generation of primary  $T_C$  cells against virus-infected syngeneic cells *in vitro* were explored. Under optimal conditions, the *in vitro* response had similar kinetics and potency to the primary response in the spleens of virus-infected mice.

Experiments with an *H-2* mutant, BALB/c-*H-2*<sup>db</sup> suggest that the molecular requirements are different for triggering precursor  $T_C$  cell induction and lysis by effector  $T_C$  cells. BALB/c-*H-2*<sup>db</sup> is a loss mutation which occurred in the *H-2D*<sup>d</sup> region of the *H-2* complex. Serological studies indicate that the *H-2D*<sup>d</sup> region apparently codes for two similar glycoproteins, *H-2D* and *H-2L* and it is the latter class of molecules which is not expressed in the mutant. Evidence reported in Chapter 5 suggests that *H-2L* might be involved in a physical association between *H-2D* molecules and viral antigens. The resulting molecular arrangement appears to be required for efficient induction of precursors of  $T_C$  cells but does not seem to be essential for target cell recognition and lysis by effector  $T_C$  cells.

Adsorption studies using virus-immune  $T_C$  cells and macrophage adsorption monolayers are reported in Chapter 6. They indicate that the binding of effector  $T_C$  cells to syngeneic virus-infected macrophages occurs only in cases where the infected macrophages display both viral and self *H-2* antigens together. This adsorption was specific for both *H-2* antigen and foreign antigen X, i.e., there was clear discrimination between *H-2*<sup>k</sup> and *H-2*<sup>d</sup> (acting as "self-markers"), and between TNP, influenza (JAP) and Sendai viral antigens (acting as foreign antigen). These results are discussed in view of current models for *H-2* restriction.

Data given in Chapter 7 show that under certain conditions  $T_C$  cells can respond to, and lyse, virus-infected allogeneic cells. Results from radiation chimeras and from neonatally tolerant mice suggest that the *H-2* antigens on radioresistant thymic cells during  $T_C$  cell ontogeny are those

which are seen as self-markers by  $T_C$  cells which respond to virus-infected cells. It is concluded that the  $T_C$  cell repertoire for receptor structures apparently has the potential to cover allogeneic H-2 markers (in respect to the H-2 type of the  $T_C$  cell) as "self-marker", but this potential is only realized by  $T_C$  cells which differentiate in a thymic environment which expresses alloantigens. Models to conceptualize the screening or selection processes exerted on progenitor  $T_C$  cells in the thymus are discussed.

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#### 1.1. Cellular basis of immune responses to viral infections

#### 1.2. Antibody responses

#### 1.3. Cell-mediated responses

### 2. ROTAVIRUS INFECTION AS A MODEL SYSTEM

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### 5. H-2 RESTRICTED CYTOTOXIC T CELL RESPONSES

### 6. ONTOGENY OF T CELLS



## CHAPTER 1

### 1.1. Cellular basis of immune responses to viral infections

The immune response to any offending antigen is conventionally divided into the humoral and the cell-mediated components; the latter can be transferred from one individual to another by immune lymphoid cells but not by serum. The two arms of the immune response are initiated by different types of immunologically reactive lymphocytes, which are derived from their origin into B (Burkitt's lymphoma or its mammalian equivalent) and T (thymus) dependent cells, which include suppressor and regulator cells. Furthermore, potentially important roles are played by macrophages (monocytes and polymorphs) and complement. The expression of an immune response is the

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In the following brief summary of immune responses to viral infections, physiological resistance factors such as interferon, body temperature, nutrition and hormones will not be included. Immune responses causing pathological changes (e.g., in the case of lymphocytic choriomeningitis virus infection) and immunosuppression to unrelated antigens caused by viruses will not be discussed.

Many viruses replicate only in a limited range of host cell types. The most striking inherited differences are seen between species of

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The immune response to any offending antigen is conveniently divided into the humoral and the cell-mediated components; the latter can be transferred from one individual to another by immune lymphoid cells but not by serum. The two arms of the immune response are initiated by different types of immunologically reactive lymphocytes, divided according to their origin into B (Bursa of Fabricius or its mammalian equivalent) and T (thymus) dependent cells, which include suppressor and regulator cells. Furthermore, potentially important roles are played by phagocytic cells (macrophages and polymorphs) and complement. Thus expression of an immune response is the net result of complex synergistic and antagonistic activities performed by a variety of cell types, possibly at different times after encountering antigen. The relative importance of different component mechanisms varies from one virus infection to another. The characteristics of an immune response to a particular virus infection are intimately related to the natural pathogenesis, the route of infection, prior antigenic exposure and many other parameters of host-virus interaction. The pathology of infectious diseases is the subject of a recent monograph by Mims (1976), in which many aspects of viral infections are dealt with in detail.

In the following brief summary of immune responses to viral infections, physiological resistance factors such as interferon, body temperature, nutrition and hormones will not be included. Immune responses causing pathological changes (e.g., in the case of lymphocytic choriomeningitis virus infection) and immunosuppression to unrelated antigens caused by viruses will not be discussed.

Many viruses replicate only in a limited range of host cell types. The most striking inherited differences are manifested between species of



animals. The genetic resistance or susceptibility to certain virus infections can be dependent upon the absence or presence of cellular receptors for the virus. The pathogenic consequences of viral infection are determined by interactions between viral and cellular genes, which affect various phases of the viral life cycle in the organism. Thus inherited differences can be manifested at several levels, some representing innate, while others involve acquired immunity. Selective deficiencies in immunoglobulins, complement components and cellular functions provide interesting information about the mechanisms of resistance. In mammalian hosts the genetic control of viral infections is polygenic. Inbred mouse strains have provided a useful model to identify genes (especially genes in the *H-2* complex of the mouse) which affect host responses to various infections (McDevitt *et al.*, 1974; Pincus and Snyder, 1975).

The available information about different mechanisms in immune responses to viral infections is often derived from *in vitro* studies. The development of appropriate culture techniques, enabling cell and tissue interactions, as well as differentiation and triggering of lymphoid cells, has contributed to the better understanding of the immune system. However, direct extrapolation from findings *in vitro* to the relevance of a particular mechanism in the intact animal can be misleading. It is obvious that *in vivo* studies have to complement *in vitro* approaches. If it can be demonstrated that a certain effect in the animal is dependent on the administration of cells or serum, careful examination of the effector mechanism *in vivo* is still required. Quite often this part of the investigation has to be left to speculation because exact determination of the processes involved is beyond experimental access.

In a response of a host animal to a viral infection, T and B lymphocytes collaborate with macrophages in the recovery process. One function of



macrophages is that of an accessory cell in both cellular and humoral immune responses (van Furth, 1975; Nelson, 1976) namely, they seem to be involved in the induction of immune responses and as accessory effector cells. Virtually all studies to define the role of macrophages in the induction of antibody production have been carried out *in vitro* (Basten and Mitchell, 1976). They demonstrate the complexity of cellular interactions involved and further investigations to elucidate the physiological significance of accessory cells in antibody production is required. At the effector level, macrophages seem to be involved in neutralizing virus particles. In the case of cell-mediated responses, macrophages have been shown to be essential participants in the initiation phase. Antigens processed by macrophages and/or presented on the surface membrane of macrophages appear to stimulate precursor T cells very efficiently. Furthermore, macrophages seem to have effector functions: immunologically specific events trigger the release of chemical mediators. These substances have a variety of biological effects, including the attraction and activation of macrophages (Blanden *et al.*, 1976a). The attraction of blood monocytes to foci of infection appears to be one of their most important functions in certain viral infections. In the case of mousepox, T cell-mediated monocyte recruitment seems to be essential for recovery.

### 1.2. Antibody responses

The development of antibodies is a characteristic response to most viral infections. It is important to differentiate between the responses of animals on their first exposure to an antigen (primary response) and on subsequent exposure (secondary response). In general, introduction of an antigen into a previously untreated animal does not elicit immediate antibody production. An inductive phase of variable length is followed by the initial appearance of IgM antibodies and usually the later appearance

of IgG antibodies. Upon re-exposure to the same antigen a secondary response occurs, which is characterized by (1) a shorter induction period than the primary response, (2) the production of very much larger amounts of antibody than are found after the primary response and (3) the production of mainly IgG antibodies. In the case of secretory IgA antibodies, very little is known about the primary and secondary responses.

Mechanisms by which antibodies may act in the infected host are discussed below. The importance of antibodies in recovery from viral infections changes very much from primary to secondary infections. If resistance to reinfection is considered, circulating antibodies are of supreme importance, because their production rate (as described above) can limit a viremia or prevent the disease. All approaches to immunoprophylaxis so far rest upon the production of antibodies, i.e., the objective of vaccination is to provide effective immunity by establishing adequate levels of antibody and above all a primed population of cells, which can rapidly expand on renewed contact with antigen. This in turn results in an accelerated antibody synthesis as compared to a primary response. Effective vaccines have been developed against many generalized viral diseases of both man and domestic animal. The WHO world-wide smallpox eradication campaign has led to a considerable reduction in the number of countries in which smallpox is endemic. Human populations are now becoming less and less immune to smallpox; in many countries smallpox vaccination is no longer required. There is speculation about the reappearance of poxviruses pathogenic to man, possibly due to animal reservoirs, e.g., the "white" poxvirus of monkeys.

In contrast, the role of antibodies in recovery from primary infection varies considerably. In most infections antibodies are produced too late to be effective. In some viral infections other factors play a greater role,



notably cell-mediated immunity and factors like interferon, but there is evidence that circulating antibodies can limit viraemia in primary infections and thus prevent spread of virus to highly susceptible target organs.

There are several possible ways that antibodies can confer protection. The mechanism of virus neutralization by antibody is still under investigation, but it appears that the attachment of one antibody molecule to a native virus is certainly not sufficient to cause neutralization. The *in vivo* importance of the three proposed mechanisms (*viz.*, coating of the virus surface, aggregation of particles, virolysis) remains to be determined (Daniels, 1975). Antiviral antibodies are thought to act by opsonizing virus particles and thus promoting their phagocytosis and subsequent digestion. Some viruses (e.g., poliovirus and influenza virus) are rapidly released from the cells in which they multiply, and then immediately exposed to the action of neutralizing antibody in their environment, which prevents the infection of neighbouring cells. Others, like herpes simplex virus, often pass directly from one cell to another. Expanding lesions can occur even in the presence of high concentrations of neutralizing antibodies. An extensive *in vitro* study by Hooks *et al.*, (1976) shows that herpes simplex virus, cytomegalovirus, vaccinia and measles are able to spread in the presence of neutralizing antibody. Coxsackievirus, encephalomyocarditis virus, vesicular stomatitis virus, mumps virus and simian virus 5 failed to spread. Complement seems to have the ability to enhance the neutralizing ability of antibody (Daniels, 1975). It is suggested that it coats the surface of virus particles and thereby causes a decrease in infectivity.

Rager-Zisman and Allison (1976) reported that in *in vivo* herpes simplex virus 1 infections antibody-dependent cell-mediated lysis of infected cells may be an antiviral mechanism.

Detailed *in vitro* examinations show that cells infected with members



of most virus groups can be lysed by antibody and complement. Thus, viruses which induce specific antigenic changes of the cell membrane are potentially controllable by this mechanism. However not all virus-infected cells can be lysed with antibody and complement and the role of complement-dependent antibody-mediated cell lysis in the immunopathology and control of virus infection *in vivo* is not yet known (Rawls and Tompkins, 1975).

The locally-stimulated secretory antibody response at mucosal portals of entry is quite independent of the systemic immunological responses. The discovery of the production and local activity of IgA antibodies has important implications for understanding the role of antibodies in all infections that are localized to mucous membranes, or have an initial stage of multiplication in mucous membranes before they spread further through the body (Tomasi and Bienenstock, 1968). Investigations by Rossen *et al.*, (1971) show that IgA found in human nasal secretions is of major importance in protection against infections with respiratory viruses.

### 1.3. Cell-mediated responses

Current understanding of the function of cell-mediated immunity (CMI) in antiviral recovery and resistance mechanisms is the result of studies in immunologically deficient patients, neonatally thymectomized or athymic animals, animals given antilymphocyte sera or immunosuppressive drugs and from cell transfer experiments (World Health Organization, 1973). Such experiments show that CMI plays a role in recovery from many virus infections (Woodruff and Woodruff, 1975). In general, sensitization of cytotoxic cells in virus infections is an early event. Often it can be detected four to six days after infection, reaching a peak before large amounts of antibodies are produced.

These differences between the kinetics of antibody synthesis and the generation of cytotoxic T cells illustrate the importance of CMI in recovery

from primary infections. As detailed above, antibodies are most effective in secondary infections. Conversely, the role of T cells in resistance to secondary infections is unknown, but may be potentially important if virus evades neutralization by antibody and succeeds in spreading from the portal of entry to crucial target organs such as the liver, the spleen or the brain.

In animal systems it has been possible to demonstrate that thymic impairment (either by neonatal thymectomy or by treatment with antilymphocyte or antithymocyte serum) can markedly increase susceptibility to ectromelia virus (Blanden, 1970), herpes simplex virus (Zisman *et al.*, 1970) and vaccinia virus (Hirsch *et al.*, 1968). Such treatments have no effect in infections with enteroviruses such as Coxsackie B-3 or flaviviruses such as yellow fever virus (Woodruff and Woodruff, 1974; Zisman *et al.*, 1971). These results imply that the generation of CMI may not be required in recovery from certain primary infections.

Observations on human patients with immunodeficiency syndromes show a similar picture. Patients with severe hypogammaglobulinaemia but normal CMI suffer from recurrent bacterial infections and have increased risk of paralytic poliomyelitis. They recover normally from smallpox vaccination and varicella or measles infection (Fulginiti *et al.*, 1968). In contrast, infants with defective CMI suffer from severe infections with vaccinia virus, herpes virus and measles.

A direct test for the involvement of cytotoxic T cells in recovery from primary virus infections comes from cell transfer experiments. The effect of immune cells in virus-infected recipients can be measured in two ways: the reduction of virus titres in target organs can be determined, or the survival rate can be scored. Such transfer experiments provide a powerful tool. Fractionation procedures allow identification of the active subpopulation of cells. Furthermore, recipient animals can be



manipulated in various ways to determine whether the transferred immune cells need to interact with particular cell populations in the recipient animal to exert their antiviral effect; e.g., prior irradiation of recipient mice to reduce blood monocyte levels. Probably the best documented CMI response in an animal model is the T cell response to ectromelia virus which causes mousepox in mice (reviewed by Blanden, 1974). This system will be discussed in detail in the next section. Similar results have been obtained with an arenavirus. Adoptive immunization of recipient mice infected with lymphocytic choriomeningitis virus (LCMV) is mediated by virus-specific thymus-derived lymphocytes (Mims and Blanden, 1972; Zinkernagel and Welsh, 1976). Protection was measured as reduction of LCMV plaque-forming units in spleen, liver and lung.

T cells from mice which had been infected with attenuated Venezuelan equine encephalitis virus (VEE) conferred protection to syngeneic animals challenged with a normally lethal dose of the same virus (Rabinowitz and Adler, 1973). However, pooled hyperimmune anti-VEE serum from guinea pigs was sufficient to ensure survival after lethal challenge with VEE. Seven-day immune cells after treatment with anti-theta serum did not show any antiviral activity, but treatment with anti-mouse gamma globulin had no effect. Transfer experiments with disrupted immune cells show that preformed cellular factors do not confer protection. Interferon has been shown to be ineffective. These data strongly suggest that T cells are involved in recovery from primary infection also in the case of an arbovirus.

In a report by Starr and Allison (1977) T lymphocytes were found to be important in recovery from murine cytomegalovirus infection. Administration of immune T cells to lethally infected mice resulted in significantly increased survival. Immune serum failed to confer protection.



Recent reports from Yap and Ada (1978) show that T cells have a protective role in the recovery of mice from influenza virus infections. Mice given a lethal dose of virus survived after administration of primary or secondary immune cells. Treatment with anti-theta and complement or separation of Ig+ve cells from Ig-ve cells clearly established that T lymphocytes are the active cells in the population. Mapping experiments and treatment with Ly sera showed that cytotoxic T cells play a major role in reducing lung virus titres (Yap *et al.*, 1978).

Three mechanisms are proposed for cell-mediated protection of hosts against virus infections: CMI responses can recruit monocytes into sites of virus multiplication; responding lymphocytes can release interferon, or sensitized lymphocytes can directly destroy infected cells which have virus-specific antigens on their surface (for review see Blanden, 1974). Zinkernagel and Althage (1977) reported experiments with vaccinia virus which provide supporting evidence for the third mechanism. Virus-immune cytotoxic T cells can effectively inhibit growth of the virus in infected target cells *in vitro* by destroying infected target cells before infectious virus progeny are assembled.

## 2. ECTROMELIA VIRUS INFECTION AS A MODEL SYSTEM

Ectromelia virus is the murine representative of the mammalian pox viruses, closely related to vaccinia and variola virus. It causes a natural disease in mice and provides an excellent laboratory model for the study of a cytopathic virus infection. The basic features of the pathogenesis of mousepox have been reviewed by Fenner (1949). The natural portal of entry seems to be abrasions on the foot-pads. Local multiplication is followed by virus dissemination *via* lymph and blood to the liver and spleen. Death is attributed to unchecked growth of virus and massive necrosis in the liver. Blanden (1970, 1971a, 1971b) carried out a series of investigations to

elucidate the relative importance of different mechanisms of recovery from ectromelia virus infection in mice. It could be shown to depend mainly on the presence of activated T cells in the circulation. These cells appear between day 4 and 6 after infection and recognize viral antigen in the liver lesions. They provoke an inflammatory cell infiltration of the necrotic areas.

Macrophages (derived from blood monocytes) appear to be the dominant cells in mature lesions. They seem to ingest and destroy infectious virus and necrotic tissue, which in turn leads to recovery. Circulating antibody can first be detected on day 7 or 8 after infection and does not seem to play a key role in recovery from primary infection. However, antibodies protect from reinfection, presumably because they can reduce infection in the visceral target organs by neutralizing the primary viraemia (Fenner, 1949).

These effector T cells generated in infected mice are cytotoxic for virus-infected target cells when tested in an *in vitro* cytotoxicity assay (Gardner *et al.*, 1974a, 1974b). The <sup>51</sup>Cr release assay has been shown to measure a T cell function, as originally defined for the allograft model by Cerottini and Brunner (1974). Activity is susceptible to treatment with anti-theta ascitic fluid and complement, is a property of Ig negative cells, is unaffected by macrophage depletion and requires cell-to-cell contact. No soluble lytic factors are detectable and complement is not required (reviewed by Blanden *et al.*, 1977a). Thus rigorous investigations have established that virus-infected target cells are lysed by effector T cells which act through direct contact with target cells and do not require auxiliary cells.

In cell transfer experiments, the antiviral activity of ectromelia virus-immune spleen cells was tested *in vivo*. The experimental protocol was as follows: Ectromelia-immune spleen cells were transferred



intravenously (i.v.) to recipients which had been infected i.v. 24h previously. Spleens and livers were removed 24h after cell transfer and titrated for virus. Ig-bearing cells (B cells) possess no detectable activity, whereas a similar dose of non-Ig-bearing cells (T cells) produced a highly significant reduction of virus titres in recipient spleens (Blanden *et al.*, 1975a).

In order to determine whether the T cells responsible for antiviral effects *in vivo* are distinct from those exerting cytotoxicity against virus-infected target cells *in vitro*, the kinetics of generation of active cells were compared (Blanden and Gardner, 1976). These two different functional assays reveal very similar kinetics. They seem to reflect the activities of the same T cell subset. Another similarity is that lysis of virus-infected cells *in vitro* and virus clearance *in vivo* (Kees and Blanden, 1976 and Chapter 2) both require that immune T cell donors share a part of an H-2 haplotype with the infected target cells or recipient mice, respectively. In both cases the essential genes map in either the *K* or *D* regions of the *H-2* complex. The above-mentioned effector functions are virus-specific, as tested against Sendai and lymphocytic choriomeningitis (LCM) virus (Doherty and Zinkernagel, 1976). Cross-reactivity has been found for the closely related vaccinia virus and ectromelia virus.

In summary, effector T cells involved in virus clearance seem to employ an antigen-recognition mechanism involving either *K* or *D* region genes, as do effector T cells which lyse target cells *in vitro*. It is most striking that T cells do not recognize virus-specified antigenic determinants alone but only in combination with H-2 determinants. This finding of H-2 restriction, first reported by Zinkernagel and Doherty (1974a) for the LCM system, has now been shown in many viral systems and is an area of very active investigation. The mouse major histocompatibility

complex on Chromosome 17 is not only of interest in the context of viral infections; its gene products are involved in many crucial interactions of immune functions. To unravel the mechanisms involved is a basic problem in cellular immunology.

### 3. THE MAJOR HISTOCOMPATIBILITY COMPLEX OF THE MOUSE

All vertebrate species so far investigated, which includes birds and amphibia, possess a major histocompatibility complex (MHC): for the mouse it is the *H-2* gene complex, for man it is the HLA system. The *H-2* complex covers a length of DNA equivalent to 0.5 recombination units (Klein, 1975). It is not known how much of the MHC codes for protein. The amount of DNA in a piece of chromosome of this size could, however, code for as many as  $2 \times 10^3$  polypeptide chains of average length. Over recent years, studies of the role of the *H-2* complex have progressed rapidly. The MHC has been shown to be extremely complex genetically, consisting of perhaps several hundred loci.

*H-2* congenic lines have been available for some time. More recently intra *H-2* recombinant strains have served to divide the *H-2* complex into various regions and subregions, which, currently are *K*, *IA*, *IB*, *IJ*, *IE*, *IC*, *S*, *G* and *D*. Each region or subregion is identified by a marker locus, which affects transplantation phenomena (*H-2K* and *H-2D*), *Ir* genes or *Ia* specificities (*IA*, *IB*, *IJ*, *IE*, *IC*), serum lipoprotein levels (*S*) or a specificity (*H-2,7*) detected predominantly by haemagglutination (*G*).

*H-2K* and *H-2D* code for major histocompatibility or transplantation antigens. They are expressed independently on the cell surface and are responsible for rapid allograft rejection (reviewed by Shreffler and David, 1975; Klein, 1975). Heterozygous animals express the alleles from both chromosomes (i.e., without dominance). The serologically defined transplantation antigens are glycoproteins with a molecular weight of about 45000 daltons. Products of each *K*- or *D*- region are characterized by a combination of one private and



several public specificities, which are all detected serologically. Private specificities are restricted to one *K* or *D* allele; public specificities are shared by several alleles and some may well represent heterogenous serological entities. Both types of specificities (private and public) are usually on the same polypeptide chain although there are now known exceptions to this rule. Lemonnier *et al.*, (1975) carried out differential redistribution studies to investigate the relationship between private and public specificities of a *D* region allele of the *H-2* complex. These data indicate that at least some of the public specificities are expressed on some of the polypeptide chains independently of those carrying the private specificity. By performing sequential precipitations with anti-*H-2* sera specific for *D* region products, two different types of molecules could be detected (Hansen *et al.*, 1977a). One showed the expected reactivity with both antisera to private and to public specificities. An additional molecule was detected which reacted only with antisera to public specificities. SDS gel electrophoresis migration patterns indicate that both products have a molecular weight of about 45000 daltons. Confirmation of these results was presented contemporaneously by Neauport-Sautes *et al.*, (1977).

When more than one property is assigned to the products of a particular region, it does not necessarily follow that these properties belong to the same gene product: the *I* region controls the expression of another set of cell surface glycoproteins with a molecular weight of approximately 30000 daltons, the so called *I* region associated on Ia antigens, (reviewed by Sachs, 1976). Parish *et al.*, (1976a, 1976b) reported that substantial quantities of low-molecular-weight Ia antigens are present in normal mouse serum. These serum Ia antigens appeared to be secreted by T lymphocytes recently activated by antigen. The function of these antigens is at present unclear, but

they seem to play a central role in mediating cellular interactions among T cells, B cells and macrophages during the immune response. Ia antigens and the immune response genes (*I<sub>r</sub>* genes) are coded in the same region. *I<sub>r</sub>* genes control the ability of an animal to respond to particular antigens; so far mainly synthetic polypeptides have been tested (McDevitt and Benacerraf, 1969). The *I* region is associated with the cooperation of the T helper cells with B cells (Katz and Benacerraf, 1975), the induction of T helper cells (Erb and Feldmann, 1975) and the adoptive transfer of delayed-type hypersensitivity (DTH) to fowl gamma globulin (Miller *et al.*, 1975).

Recently a study of ultimate lifespans in congenic mice (Smith and Walford, 1977) suggests that the MHC is one of the gene systems involved in the control of ageing, but whether an immunological function is concerned with this phenomenon is unknown. Apart from immunological functions, serum lipoprotein and complement levels are also under the control of the *H-2* complex.

#### 4. H-2 MUTANTS

Mutations affecting the *H-2* complex have been recently studied in several different systems. They have proven to be of great interest in studying T cell interactions in graft-versus-host reactions (GVHR), in mixed lymphocyte reactions (MLR) and in cell-mediated lysis (CML). Methods used to screen for *H-2* mutations are based on grafting procedures; serological tests do not seem to be feasible. However, to detect mutations in regions other than *H-2K* or *H-2D*, serological methods would have to be used, unless *I* region differences evoke detectable skin-graft rejection. This has been shown to be the case for the *IA* and *IC* regions. In the mouse, spontaneous mutations have arisen, others have been induced chemically or by irradiation. The three mutation types (gain, loss, gain-and-loss) can be distinguished by the different pattern of graft rejection within groups



of mice. Of the  $H-2$  mutants described so far, the most widely studied are those occurring within the  $K$  region of the  $H-2^b$  haplotype and those occurring in the  $D$  region of the  $H-2^d$  haplotype. A mutation is defined as an inheritable alteration of the genetic material not caused by recombination. If the alteration affects a single gene it is called a point mutation (Klein, 1975). A point mutation is not to be confused with base-pair switches known from bacterial or viral genetics. The most unstable allele known in the MHC of the mouse is  $H-2K^b$ . Melvold and Kohn (1975) tested an approximately-equal number of  $H-2^b$  and  $H-2^d$  genes and found nine independent mutations among 26316 mice. Eight of the nine mutations were found to be of the  $H-2K^b$  allele. The calculated mutation rate of the  $H-2K^b$  gene is therefore  $5.5 \times 10^{-4}$  per generation, which is by far the highest reported mutation rate in a mammal. An explanation for this extraordinary high rate has yet to be found. It is likely that  $H-2K^b$  is a single locus, not a cluster of many loci, since the above mentioned eight mutant alleles failed to complement each other. Another possibility is that the mutations are regulatory rather than structural. This is, however, difficult to visualize because all eight mutants are of the gain-and-loss type. A recent review by McKenzie *et al.*, (1977) shows that these  $H-2$  mutants provide important clues to the understanding of  $H-2$  genetic structure.

One of the best characterized mutants is B6.C- $H-2^{ba}$ , (formerly called B6.C(Hz1)), detected by Bailey and Kohn (1965), and analyzed by Bailey *et al.*, (1971). It is a spontaneous mutation which arose in a (C57BL/6 x BALB/c)  $F_1$  female and was subsequently placed on the C57BL/6 background by repeated backcrossing and selection for the mutant phenotype. Further investigation indicated that the "K-end" of  $H-2^b$  was the site of the  $ba$  mutation (Bailey *et al.*, 1971; McKenzie *et al.*, 1976). More precise genetic mapping of the mutation locus is not possible at present because of

the lack of recombinants of  $H-2^b$  occurring between  $H-2K$  and the  $IA$  regions. Grafts exchanged between C57BL/C and  $H-2^{ba}$  are all rejected, indicating that the mutation is of the gain-and-loss type. This is also reflected in three other T cell-mediated tests (MLR, CML and GVHR). The striking feature of  $H-2^{ba}$  is that cross-immunizations with the wild type have been unsuccessful in producing antibodies; at the most, small quantitative differences in the expression of  $H-2K.33$  could be detected by some laboratories, but not by others (reviewed by McKenzie *et al.*, 1977). The finding that T cell activation occurs without any major detectable serological difference seems to indicate that B cell and T cell activating determinants are different or that their receptor specificity is different, in analogy to the hapten-carrier situation. The effect of the  $ba$  mutation on T cell antiviral function *in vivo* has been investigated (see Chapter 2).

Another spontaneous mutant is BALB/c- $H-2^{db}$  (Melvold and Kohn, 1976). It is a loss mutation in that  $H-2^{db}$  mice will reject BALB/c grafts, but there is no rejection in the reverse direction. Complementation studies have localized the mutation to the same locus in  $H-2D$  as B10.D2- $H-2^{da}$  (McKenzie *et al.*, 1977). There are serologically detectable changes in the mutant:  $H-2^{db}$  lacks the known specificities of the  $H-2.28$  family and produces an anti- $H-2.28$  antibody upon immunization with BALB/c. This public specificity seems to be lost whereas no alteration in the private  $H-2D^d$  specificity  $H-2.4$  could be demonstrated. The structural relationship between these two moieties has been investigated by Hansen *et al.*, (1977b). Immunological studies indicate that the  $H-2D^d$  region apparently codes for two similar proteins, each of about 45000 molecular weight. One of these react with a serum detecting the private  $H-2D^d$  specificity  $H-2.4$ . It can be demonstrated in wild type and in the mutant. The class of molecules, detectable in the wild type strain but missing in the mutant, does not bear private specificities,



but does react with antibodies contained in anti-H-2.28 sera. This molecule has been designated D' by Hansen *et al.*, (1977b). These investigations confirm earlier findings indicating that the H-2D<sup>d</sup> region codes for two similar antigens on the cell surface (Lemonnier *et al.*, 1975; Hansen *et al.*, 1977a) (see above). At present it is unclear which molecule D or D', or both, bear the public specificities currently assigned to the D region. Recombinants that separate D from D' would be required for accurate assignment of specificities to these molecules. The role of D' molecules in ectromelia virus-induced antigenic changes has been examined in Chapter 5.

Knowledge of the molecular structure of H-2 and Ia antigens has long been sought as a key to understanding the functions of these molecules. All major components of living matter (nucleic acids, lipids, carbohydrates, proteins) have been implicated at one time or another as carriers of H-2 antigenicity. The gene products of the K and D region have the following general chemical properties (Silver and Hood, 1976): (1) They are hydrophobic cell surface glycoproteins, about 45000 daltons in molecular weight; (2) The antigenic specificities appear to be carried by the protein and not by the carbohydrate portion of this molecule; (3) The N-terminal end of the molecule extends away from the cell surface, whereas the C-terminal portion is embedded in the membrane; (4) The K or D gene products are noncovalently associated with murine  $\beta_2$  microglobulin on the cell surface. The  $\beta_2$  microglobulin, a polypeptide of about 12000 daltons, is an immunoglobulin-like molecule, whose determining gene is not linked to the MHC.

Peptide mapping revealed considerable differences between products of

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The correct name for the locus coding for D' is now H-2L, coding for H-2L protein (Morgan *et al.*, 1978).

K and D genes (Brown *et al.*, 1974) and between products of alleles of the same gene (Brown and Nathenson, 1977) as comparisons between H-2K<sup>ba</sup>, H-2K<sup>bd</sup> and H-2K<sup>b</sup> glycoproteins showed. A significant degree of diversity between peptides of the mutant D<sup>da</sup> molecule and the wild type molecule H-2.4 could be detected by Brown *et al.*, (1978), which indicates that the primary amino acid sequence determines the antigenic specificities. However, altered carbohydrate side chains (perhaps as a consequence of altered amino acid sequences at the site of carbohydrate attachment) could not be excluded.

Recently microsequencing techniques have been employed on the N-termini of H-2 K and H-2 D antigens. Comparable data on the classical transplantation antigen of man are also available. So far, preliminary amino acid sequences data are only published for the first 25 residues. (An H-2 K or H-2 D molecule may consist of about 250 to 300 amino acid residues.) On the basis of these very limited results it is suggested that human and murine transplantation antigen have descended from a common ancestral gene.

## 5. H-2 RESTRICTED CYTOTOXIC T CELL RESPONSES

Zinkernagel and Doherty (1974a) originally observed that the splenic T lymphocytes from mice acutely infected with the arenavirus lymphocytic choriomeningitis (LCM) virus showed optimal cytolytic activity *in vitro* only against LCMV infected targets which possessed the same H-2 gene complex as the effector cells. A similar result was obtained independently by Shearer (1974) using lymphocytes sensitized *in vitro* against trinitrophenyl (TNP) modified syngeneic cells. Furthermore, the phenomenon has also been observed by Wainberg *et al.*, (1974) for the T cell response to Rous sarcoma virus in chickens.

These initial results could be confirmed for ectromelia virus (Blanden *et al.*, 1975b; Gardner *et al.*, 1975) and have now been extended to include a diverse group of viruses: Coxsackievirus B-3 (Wong *et al.*, 1977b),



influenza virus (Yap and Ada, 1977), Sendai virus (Doherty and Zinkernagel, 1976), vaccinia virus (Koszinowski and Ertl, 1975), vesicular stomatitis virus (Hale *et al.*, 1978) group A arboviruses (A. Muellbacher, personal communication), and adenoviruses (Uetake and Inada, 1977). Until recently attempts to demonstrate herpes simplex virus (HSV)-specific cytotoxic T lymphocytes had failed. Pfizenmaier *et al.*, (1977) could demonstrate that murine lymphocytes sensitized *in vivo* differentiate into effector cells after further culturing for 72 hrs. The authors suggest that the *in vivo* state of low cytotoxic T lymphocyte activity might be under the control of cyclophosphamide-sensitive T suppressor cells. It is of interest to note that Coxsackie B virus-infected mice generate a cell-mediated immune response, since the virus is a member of the picornavirus family, small RNA-containing viruses that lack envelopes and do not contain lipid. They are shed from the cell surface during the early cytopathologic process through vacuoles which fuse with the plasma membrane and are also released from the cells at the time of lysis. However, the results by Wong *et al.*, (1977a) indicate that the Coxsackievirus infection elicits changes in cell surface membranes recognizable by T cells. So far, viral antigen could not be detected on the membranes of infected cells by immunofluorescent staining, and lysis did not occur after treatment with antiserum and complement.

In several of the above mentioned viral systems it has been observed that the early response (around day 3 of infection) lacks viral specificity; both infected and uninfected target cells are lysed (Pfizenmaier *et al.*, 1977; Wong *et al.*, 1977a; Welsh and Zinkernagel, 1977; Blanden and Gardner, 1976). The later response is specifically directed against cells infected with the virus used for immunization. This early transient production of cytotoxic cells capable of killing uninfected syngeneic target

cells is not yet understood. The possibility that the lysis could be attributed to infectious virus carried over with the immune spleen cells could be excluded in all reports. There seems to be disagreement as to the nature of this cytotoxic cell. Some authors suggest that the cytotoxic process is largely dependent on T cells (Blanden and Gardner, 1976), whereas others claim that the treatment with anti-theta serum and complement has no effect (Welsh and Zinkernagel, 1977). Generally the activity is observed on a variety of targets, syngeneic, allogeneic or even xenogeneic to the effector cells. The possible relevance of these findings is as yet unclear (for discussion see Blanden and Gardner, 1976). There may be a multi-clonal cell response due to the sensitization with virus, whose function in the context of the immune response remains to be determined. It is possible that these apparently self-reactive cells play an accessory role in the generation of virus-specific T cells. If that were the case such a function could be detected by using adsorption techniques (e.g., on day 3 after infection). The effect of removing these cells on the generation of virus-specific T cells would have to be assessed after further culturing the non-adherent cells, depleted of apparently self-reactive cells. Recently presented data (Butchko, 1978) clearly demonstrate that H2N2 influenza viruses are mitogenic for lymphocytes. Maximum <sup>3</sup>H-thymidine incorporation occurred after 2 days of culture and both T and B cells were stimulated. The study did not include a cytotoxicity test. Such mitogenic action supports the idea of multiclonal T cell activation occurring during some viral infections. If effector cells are harvested on day 5 or 6 after infection, virus-specificity can be clearly demonstrated. The same is true for cells sensitized *in vitro* with virus-infected syngeneic cells, (see Chapter 4 and Blanden *et al.*, 1977b; Jung *et al.*, 1978).

Successful cellular interaction (tested in the <sup>51</sup>Cr assay) occurs only



if donors of immune T cells and virus infected target cells share genes mapping at, or near *H-2K* or *H-2D* ; identity at either locus is sufficient. Differences in minor H antigens, at the M locus or in the *I* or *S* region of the MHC are apparently irrelevant. Furthermore, presence of a completely unshared H-2 chromosome (in  $F_1$  interactions) or of different *H-2K* or *H-2D* genes (in H-2 recombinant experiments) has no obvious deleterious effect.

The availability of *H-2* mutant mice has allowed further definition of the requirement for H-2 compatibility. LCM, ectromelia or vaccinia virus-immune spleen cells of *H-2<sup>b</sup>* mutant mice carrying a point mutation in the *K* region (*H-2<sup>ba</sup>*, *H-2<sup>bf</sup>*) cannot lyse infected target cells expressing the wild type *H-2<sup>b</sup>* allele and *vice versa* (Blanden *et al.*, 1976b; Zinkernagel, 1976). This restriction holds as well for effector T cell function *in vivo*, (see Chapter 2 and Kees and Blanden, 1976). These data suggest that the effect the *K* region has on T cell recognition of syngeneic virus infected cells is mediated through a single genetic element which is also responsible for the alloantigenic patterns recognized by effector T cells of a different H-2 type. In contrast Forman and Klein (1977) demonstrated that crossreactivity between mutants and wild type occurs with cytotoxic T cells sensitized to TNP modified syngeneic cells. There are two ways of viewing this discrepancy: one is to consider the possibility that H-2K molecules have at least two antigenic determinants and that *H-2K<sup>b</sup>* and *H-2K<sup>ba</sup>* share one, but not the other. The shared determinant could be recognized with TNP and the unique determinant could be recognized with viral antigens. The problem then is to explain why this should be so, and no obvious explanation has been found as yet. A second way of viewing the problem is to consider the possibility that the subsets of T cells that respond to virus-infected self cells have greater specificity for self H-2K antigens than the subsets that respond to TNP-

modified self cells. A precedent for this thinking exists in findings that H-2 restriction (i.e., specificity for self H-2) is more rigorous for viral than for TNP systems (Burakoff *et al.*, 1976).

H-2 restricted cytotoxic T cells have been demonstrated in several experimental tumour systems. Blank *et al.*, (1976) immunized mice with H-2 compatible cultured tumour cells which were induced *in vivo* by Friend leukemia virus (FLV). Mice immunized with murine sarcoma virus (MSV) generate cytotoxic cells, as reported by Gomard *et al.*, (1976) and Holden and Herberman (1977). H-2 compatibility between donors of cytotoxic T cells and the target cells was necessary for fully efficient immune cytolysis. Effector cells generated by sensitization of lymphocytes with simian virus 40 (SV40)-transformed fibroblasts *in vitro* also showed H-2 restriction (Warnatz and Krapf, 1976; Maki and Howe, 1976). Both groups report the extraordinary finding that lymphoid cells are capable of responding to allogeneic SV40-transformed fibroblasts without concurrent response to untransformed allogeneic cells. The authors suggest that transformation of fibroblasts by SV40 results in a significant reduction in the expression of normal histocompatibility antigens on the surface of the cells. These findings are in contrast to the experiments reported in Chapter 7 where analogous tests were carried out in the viral system.

Recent reports by Gomard *et al.*, (1977) and Blank and Lilly (1977) indicate that the products of only some H-2K or H-2D regions are involved in the interactions between cytotoxic T cells and tumour target cells induced by FLV or MSV. Cytotoxic T cells generated against group A arbovirus infected cells give similar results (A. Muellbacher, personal communication). A specific association of virus products with certain MHC molecules on the cell surface or, alternatively, a specific failure to associate, could explain the observed results.



Genes in the *K* and *D* region also determine the susceptibility of target cells to lysis by cytotoxic T cells which have been immunized to minor (non-*H-2* coded) antigens (Bevan, 1975). Experiments by Gordon *et al.*, (1977) demonstrate that the T cell-mediated cytotoxic response to the male specific antigen (H-Y) is restricted to the *K* and/or *D* end of the *H-2* complex. In the latter case the production of cytotoxic cells could be shown to be under the control of *Ir* genes (Hurme *et al.*, 1978). A regulatory role for *Ir* genes has been shown for the TNP model (Schmitt-Verhulst and Shearer, 1975).

The *in vitro* secondary response to ectromelia virus infection has been characterized in detail by Gardner and Blanden (1976) and Pang and Blanden (1976a). Analysis of the genetic requirement for virus-immune T cell responses revealed that sharing *K* or *D* region between infected stimulator cells and donors of memory responder cells was sufficient for induction of the cytotoxic T cell response (Pang and Blanden, 1977). Therefore, *H-2* restriction holds not only at the level of expression of lytic activity but also for induction of secondary effector cells. If the infected stimulators share only the *I* region with the responders, highly significant levels of <sup>3</sup>H-thymidine incorporation is induced but no significant cytotoxicity can be detected. These data indicate that a major proliferative response occurs in a cell subset other than cytotoxic T cells. The cell subset involved seems likely to be an amplifier T cell responding to antigenic patterns dependent on *H-2I* genes and viral genes, since an amplifying effect of cells of this type on the cytotoxic T cells could be found (Pang and Blanden, 1977). These findings agree with those described for the generation of cytotoxic T cells against allogeneic stimulators. The participation of at least two subsets of T cells in the initiation of cytotoxic T cells against allogeneic cells *in vitro* has been postulated by several investigators

(Alter and Bach, 1974; Engers and MacDonald, 1976). It is suggested that an optimal response depends on cooperation between amplifier *Ly* 1 and prekiller *Ly* 23 cells (see next section).

Cytotoxic T cells sensitized to mouse alloantigens are usually specific for antigens coded for by the *K*- or *D*-end of the MHC. Co-cultivation of cells with different *I* regions results in strong cell proliferation (MLR), but recent reports show that *I* region coded antigens can generate a cytotoxic T cell response too, although at a much lower level than the anti H-2K or anti H-2D responses. Serum blocking studies support the notion that Ia antigens serve as targets in the *in vitro* assay (Klein *et al.*, 1977). However, so far no role for Ia antigens has been found in the recognition of viral or minor H antigens by "H-2 restricted" syngeneic cytotoxic T cells.

Several models have been proposed for the observed H-2 restriction in T cell interactions with chemically modified or virally infected cells or cells expressing minor transplantation antigens or H-Y antigens. Originally two tentative models to explain the H-2 restricted interaction between T cells and virus-infected cells were proposed (Zinkernagel and Doherty, 1974b): "Altered self" or "interaction antigen", and "physiological interaction". The latter model requires that T cells have two receptor types: one is clonally expressed and recognizes virus-specified antigenic determinants. A second "physiological" interaction between H-2 coded self-recognition structures on T cells (present on all T cells) and virus-infected cells (present on syngeneic, not on allogeneic cells) is necessary to promote binding sufficient for T cell stimulation or target cell lysis. At present, the evidence suggests that the physiological interaction model is probably not correct. Experiments supporting this view are discussed by Blanden *et al.*, (1977a). The alternative model has now two variants (for review see Blanden *et al.*, 1977a; Blanden and Ada, 1978; Zinkernagel and Doherty,



1977; Janeway *et al.*, 1976a; Langman, 1977). The first holds that this phenomenon reflects recognition of a complex antigen, formed by foreign antigen (viral, haptenic etc.) and self H-2 products by a single T cell receptor (single receptor model). In contrast, the dual recognition (also called dual receptor) model holds that individual T cells express receptors for self H-2 components in addition to receptors for foreign antigen. In more general terms: T cells employ two different, clonally expressed antigen receptors. Many experiments have been attempted to resolve these two alternatives. Thus, the debate centres on two issues: (1) What determinant(s) on target or stimulator cells are recognized by T cells and what are the contributions of the host cell genome and the viral genome? (2) Are there one or two classes of recognition structures present on cytotoxic T cells?

Various approaches have been used for the isolation and analysis of cell surface receptors. These include radioiodination of exposed membrane proteins, serological precipitation systems, isolation of surface molecules complexed to antisera etc. There are many problems in the solubilization and isolation of membrane proteins, particularly those of an integrated nature. A crucial difficulty in immunological studies has been the specificity of the antisera used and the cell type used for the isolation procedure. These difficulties might explain some of the divergent findings regarding receptors in membranes of T cells. Antigen-binding receptors on B lymphocytes seem to be comparatively conventional immunoglobulin molecules. There is controversial evidence that the T cell receptor is also a special immunoglobulin (reviewed by Marchalonis, 1976). However, many reports have been published indicating that T cells normally fail to express on their outer surface endogenous, conventional immunoglobulin molecules (reviewed by Binz and Wigzell, 1977a).

A different approach to the study of the T cell receptor makes use of antiidiotypic antibodies (reviewed by Binz and Wigzell, 1977a). The data suggest that all idiotypic determinants found on T cell receptors are also present on corresponding B cells, but not the reverse. This would indicate that the two lymphocyte groups are using some common variable (V) genes in the creation of their respective antigen-binding receptors. It was shown that the idiotype-positive, antigen-specific receptors found on or released by T cells are products synthesized by these very cells. Precise determination of the V genes shared between T and B cells is still lacking.

A lack of self-tolerance to the idiotypic T cell receptors has been demonstrated. This has allowed the induction of specific tolerance towards transplantation antigens *via* autoimmunization procedures, using the individual's own receptors. This latter approach may become a very useful tool in analyzing T cell receptors even further (see Concluding Discussion).

The specificity of cytotoxic T cells can be tested directly by measuring their relative ability to lyse a panel of target cells in a  $^{51}\text{Cr}$  assay. The target cells can either be of different H-2 types and/or infected with various viruses or chemically modified, but if the T cell population is a mixture of subsets of different specificities then other tests are required to resolve whether individual cells are specific or crossreactive. Methods to approach such questions include competitive inhibition of lysis using either unlabelled cells or purified cell-free antigens, and adsorption of the cytotoxic cells on cellular or inert immuno-adsorbents expressing the appropriate antigenic specificities, (see Chapter 6). Another attempt to separate subsets of T cells has been recently successful: the polyacrylamide culture system of Marbrook and Haskill (1974) was adapted for the segregation of individual clones of cytotoxic lymphocytes (Ching *et al.*,



1977). This system allows precursors of cytotoxic T cells to become segregated during culture, so that the responding effector cells can be harvested as separate clones.

Studies to determine the fine specificity of T cells in respect to virus-coded antigens on stimulator or target cells have been carried out by Braciale *et al.*, (1978) using the influenza virus model. A relatively new approach to analyse antigens recognized by T cells makes use of temperature-sensitive virus mutants. Hale *et al.*, (1978) demonstrated that the expression of G protein (the single surface glycoprotein) of vesicular stomatitis virus on the cell surface of target cells is necessary for killing; the expression of matrix protein M did not have any influence on the lysis of target cells. Positive identification of the antigens recognized by T cells requires more direct evidence, such as the demonstration that a purified antigen binds specifically to T cells or elicits or inhibits the development of these T cells, possibly after incorporation of the purified products into artificial membrane-bilayers (Curman *et al.*, 1978).

To further analyze the role of H-2 products, different aspects of H-2 restriction have been investigated. As indicated above the use of H-2 mutants has been very fruitful. An established cell line of murine teratoma cells (F9) lacks serologically detectable H-2 antigens by available criteria. Such cells have been tested for their capacity to act as stimulator or target cells for T cells. Zinkernagel and Oldstone (1976) report that MHC antigens do not seem to be involved in viral absorption, penetration or release, based on results with LCMV and vaccinia virus-infected F9 cells. Virus immune T cells generated *in vivo* (i.e., with stimulator cells expressing H-2 antigens) failed to lyse virus infected F9 cells. Similar results have been reported by Doherty *et al.*, (1977) using the same viral system and by

Forman and Vitetta (1975) testing T cells sensitized to TNP-modified cells. However Wagner *et al.*, (1978) could demonstrate that cytotoxic T cells can lyse H-2 negative F9 cells if primed to them. F9 target cells which were lysed by anti-F9 immune T cells were not affected by anti-H-2<sup>ba</sup> T cells. These data imply that the cytotoxic effect of sensitized T cells can take place in the absence of H-2 self markers. It is likely that the F9 antigen on these cells acts as an antigenic analogue to H-2 gene products. This in turn raises the question of whether it is possible to induce F9 restricted virus- or hapten-specific T cells.

Other approaches to characterize the role of H-2 products in T cell recognition will be dealt with in Chapters 7 and 8.

## 6. ONTOGENY OF T CELLS

The fact that T cell tolerance to self is apparently established during ontogeny, while some recognition of self H-2 antigens by T cells occurs, warrants a closer look at the known facts about T cell ontogeny. It is generally accepted that the thymus does not participate directly in immune reactions but is of vital importance in development and maturation of cell-mediated immune responses in peripheral lymphoid tissue. Although the central role of the thymus in immunity was discovered no more than 15 years ago (Miller and Osoba, 1963), an extraordinary number of publications dealing with the organ itself as well as with its cellular "products", the so-called thymus-derived cells (T cells), has since appeared. It is now well established that T cells are implicated in most aspects of immunity: either as direct effector cells (cytotoxicity phenomena, secretion of various mediators of delayed hypersensitivity) or as regulatory, helper or suppressor cells, in humoral and cell-mediated immune responses. Many of these actions involve the secretion of humoral mediators by sensitized T cells,



both antigen specific and nonspecific.

The removal of the thymus has variable effects on the development of humoral and cell-mediated responses depending on the species and the time of surgery. Immunological reactivity is generally not affected if thymectomy is carried out in adult life. Small laboratory rodents thymectomized shortly after birth often exhibit striking deficiencies, particularly in cell-mediated immune responses. A decrease in circulating lymphocytes and a severe impairment of graft rejection can be observed (Miller, 1961); mice cannot become sensitized to contact-sensitizing agents (de Sousa and Parrot, 1969) or make antibodies to a number of (T dependent) antigens (Humphrey, 1964).

In contrast to these findings, results with sheep raise the possibility of an alternative pathway of development of immunological responsiveness. Lambs can be thymectomized *in utero* after 50 days of a 150 days gestation period. These lambs are born with lymphoid tissues depleted of lymphocytes, the extent of this depletion varying from partial to severe. Nevertheless, the newborn animals subsequently develop a lymphoid system which, though depleted, appears to function in an almost normal fashion. It seems that in sheep the thymus might not be a unique location for development of immunologically competent lymphocytes which mediate CMI. Extra-thymic sites in athymic lambs may generate cells equivalent to the so-called thymus-derived lymphocytes by a sluggish process requiring months for complete reconstitution (Morris, 1973).

The following summary will be limited to the key aspects of T cell ontogeny in the mouse; no attempt is made to exhaustively review the literature. Although there is general agreement that the generation of T cells from undifferentiated stem cells is strongly thymus-dependent, there are still many unanswered questions concerning the graded steps of this

differentiation, particularly within the intrathymic environment. The effects of several thymic factors with apparent functional activity have been a matter of controversy during the last few years. There is evidence that the thymus gland does indeed produce humoral factors capable of modifying various *in vitro* and *in vivo* functions of thymocytes (for review see Bach and Carnaud, 1976). However, the understanding of thymic humoral functions is complicated by the fact that a number of different active principles from the thymus gland or from the blood have been isolated by various groups of investigators, each of these factors exhibiting different activities in a range of *in vitro* assays (van Bekkum, 1975).

The thymus is the site of a remarkable degree of cellular proliferation, differentiation and traffic. It is a bilobate organ, made up essentially of epithelial cells and large numbers of lymphocytes. In each lobe of the thymus four distinct regions can be recognized on the basis of structure and cellular populations: the outermost or subcapsular cortex, the inner cortex, the medulla and the perivascular tissue space, surrounding larger medullary blood vessels (Clark, 1973). The concentration of lymphocytes is higher in the cortex than in the medulla. The cortical cells appear to be the most mitotically-active of all haemopoietic cells, the mean cell cycle time has been calculated to be 6 to 7.5 hours (Metcalf and Moore, 1971).

Development of lymphoid cells within the thymus is characterized by the expression of unique cell markers (Raff, 1971; Lance *et al.*, 1970). The most prominent marker is the theta antigen (Thy.1). Lymphoid cells can be detected in the embryonic mouse thymus on day 12 of gestation, (for review see Stutman, 1977). The determination of time of appearance of certain surface antigens depends on the sensitivity of the serological methods used. When tested for Thy.1, 14-day-old embryonic thymus was consistently negative (Owen and Raff, 1970) although a high proportion of cells



became positive after 4 days in culture. The differentiation of embryonic stem cells to thymocytes is accompanied by other surface antigenic changes (reviewed by Owen, 1972), like TL and the (more recently used) *Ly* series. An important recent development was the demonstration that haemopoietic cells of adult or embryonic origin can express T cell markers after incubation *in vitro* with a variety of agents, e.g., thymic hormones or mitogens (Bach *et al.*, 1971; Cohen and Patterson, 1975). Functional manifestation of T cell properties seems to have more specific requirements. However, none of the experiments formally rule out the possibility that the commitment of the stem cell to T cell differentiation is a thymus-independent or prethymic event. Furthermore, the possibility that thymic humoral factors play a physiological role in subsequent maturation of T cells in the periphery still remains, (see below).

The homozygous "nude" (nu/nu) mouse (Flanagan, 1966; Pantelouris, 1968) has been widely acclaimed as an animal in which the effects of having no thymus-dependent functions can be studied. These congenitally athymic mice do not reject allogeneic skin grafts or heterografts from other rodents, nor from avian, reptilian and human donors. Antibody responses to thymus-independent antigens are not impaired, but responses to T-dependent antigens are depressed (Rygaard and Povlsen, 1976). However, there is enough evidence (Pritchard and Micklem, 1974) for the presence of a few cells with at least some T cell characteristics to warrant care in experiments which might be sensitive to slight contaminations with T cells. The T cell immunodeficiency of the nude mouse is essentially due to an abnormal morphogenesis of the epithelial compartment of the thymus. Nude mice have precursors of the cell lineage, which can repopulate the thymus of a normal mouse, differentiate, migrate to the secondary lymphoid organs of the nude and restore T cell-dependent immunity (reviewed by Loor, 1977). Experiments with thymuses in

diffusion chambers suggest that the actual traffic of prethymic precursors through the organ is mandatory and therefore that the humoral influence alone is not sufficient.

Physical properties of T lymphocytes can be used for separating them into subpopulations, (for review see Shortman, *et al.*, 1975). A range of methods is required to characterize subsets of cells: separation on the basis of cell buoyant density (which reflects average chemical composition), sedimentation velocity (which reflects primarily cell size) and electrophoretic mobility (which reflects cell surface charge). Based on such investigations a model of T cell development in the thymus has been proposed. The essential features of the model are independent pathways for high theta cells and for low theta cells, with separate dividing cell compartments. Low theta cells are apparently not derived directly from the high theta thymocytes, as proposed by other authors. The low theta cells leave the thymus and are direct progenitors of the active, low theta peripheral T cells, which finish maturation in the periphery. The fate and function of the high theta lineage is not clear. These cells might leave the thymus to form a very transient high theta population in the periphery, which never accumulates to readily detectable level, and either transforms rapidly or dies there. Metcalf's data (1966a, 1966b) together with results from Shortman *et al.*, (1975) about the behaviour of thymocyte subpopulations in cell culture suggest that these high theta cells die within the thymus. Further indication for cell death in the thymus comes from considerations about cellular proliferation in the organ. Large numbers of thymic lymphocytes are continually produced, which apparently are never released into the secondary lymphoid tissue. It is conceivable that this high theta population represents self-reactive cells which are directed into a sterile pathway. A selection process based on the distinction between self and non-self, leading to the elimination of



"forbidden" clones has been proposed by Jerne (1971). A small, self-reactive subpopulation of T cells has been detected in mouse thymus by Howe *et al.*, (1970) and von Boehmer and Byrd (1972). The latter study shows that the MLR response of CBA thymus cells to syngeneic Mitomycin-C -treated spleen cells (measured by thymidine incorporation) is very high in neonatal animals. The syngeneic response decreases with increasing age of the mice, whereas the response to allogeneic cells remained high with ageing (von Boehmer and Adams, 1973). The authors propose that the reaction to syngeneic spleen cells depends on a new H-2 self-antigen, since the apparently-recognized antigen developed in the spleen with age. It is of interest that a positive reaction could be demonstrated with CBA and BALB/c but not with C57BL or C57BL/6J thymus cells. CBA x C57BL cells responded with high values of thymidine incorporation to CBA spleen but not to C57BL spleen cells. Direct cell contact is required for the syngeneic MLR and soluble factors released by spleen cells into the culture medium are not responsible for the observed thymidine incorporation (von Boehmer, 1973).

Several investigators have directly demonstrated the emigration of thymic lymphocytes to the periphery. The two techniques used are to some extent selective for subpopulations of cells under analysis. *In situ* labelling with <sup>3</sup>H-nucleosides followed by autoradiographic and liquid scintillation tests only identifies cells which were DNA-synthesizing in the thymus. The use of thymus grafts from donors possessing distinctive chromosomal markers allows detection of mitotic cells in secondary lymphoid tissue, (reviewed by Cantor and Weissman, 1976). Most experiments support the idea that mature lymphoid cells have a clear-cut migration pattern and are not distributed at random in the peripheral lymphoid organs. The question as to what factors influence T cell migration is not yet answered. The thymus most probably exports T cells that differentiate further in the

periphery, possibly regulated by thymic humoral factors, (Stutman, 1977). The question arises whether there is a precursor T cell which may give rise to cells capable of mediating the complete range of T-dependent responses, e.g., cytotoxic responses to alloantigens (Cerottini and Brunner, 1974), responses to viral and bacterial infections (Blanden, 1974), helper functions (Transplantation Rev., 1969), suppressive effects on the production of antibody (Gershon, 1974) or other T cells, graft-versus-host (Cantor, 1972) and delayed-type inflammatory responses (David and David, 1972).

The three main characteristics of the T cells in the peripheral pool are that: (1) they recirculate repeatedly between blood and lymph, (2) they are incapable of migrating back to the thymus and (3) they possess T cell surface markers. The immunologically competent recirculating T cells are in general long-lived lymphocytes. They are less sensitive to anti Thy.1 serum than the major thymocyte population but seem to express more H-2 antigen than the latter (Aoki *et al.*, 1969). The relevance of this finding in respect to potentially self-reactive T cells in the thymus and in the periphery remains to be determined.

Several lines of evidence argue in favour of the existence of T cell sub-populations which differ according to their markers and functions. The study of T cell heterogeneity has developed rapidly in the past five years. Most of the described "systems" have been associated with either a  $T_0-T_1-T_2$  (Bach and Dardenne-) or a  $T_1-T_2$  (Raff and Cantor-) terminology. In a collective effort different investigators attempted to compare these subsets and have shown some similarities and discrepancies (Bach *et al.*, 1975). It is however not clear whether the T cell subsets that have been described represent different stages in T cell maturation or whether they are products of separate and parallel T cell lines, even if derived from a common precursor. The Ly data (see below) seem to support the latter hypothesis.



A very useful marker for subpopulations of peripheral T cells is the *Ly* group of alloantigens described initially by Boyse *et al.*, (1968). Each locus (1, 2 or 3) is expressed as one of two alleles (1 or 2). All thymocytes as well as a portion of peripheral lymphocytes bear *Ly* antigens. Using sera detecting different *Ly* antigens (together with rabbit complement), three major T cell subclasses could be detected, (reviewed by Cantor and Boyse, 1977)<sup>2</sup>. It is very important to note that the titre as well as the procedure used for *Ly* sera treatments are critical. Thus, unless experiments are carried out under optimal conditions, results can be criticized on these grounds. *Ly* 123 cells seem to be less mature than *Ly* 1 and *Ly* 23 cells, and appear first during ontogeny. It is most likely that these cells are short-lived precursors which may give rise to either *Ly* 1 or *Ly* 23 cells. *Ly* 1 cells appear to be especially activated in the presence of *I* region differences and seem to be programmed to help or amplify the functional activity of other cells, e.g., B cells, macrophages and T cells of the *Ly* 23 type in MLR. They also account for the major portion of the proliferative reaction in MLR, but do not themselves directly contribute to the cytotoxic effector cell pool. *Ly* 23 cells account for approximately 5 - 10% of the total peripheral T cell pool. Cytotoxic activity to allogeneic cells (preferentially to the *H-2K/D* regions) is mediated by *Ly* 23 cells and cells of the same *Ly* phenotype appear to have the capacity to suppress both humoral and cell-mediated immune responses. However, this might not be generally true. It remains to be seen if all murine cytotoxic T cells are of the *Ly* 23 phenotype or if it is possibly dependent on the mouse strain used. Shiku *et al.*, (1975)

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T cells expressing all three *Ly* markers are referred to as *Ly* 123 cells; accordingly, T cells expressing *Ly* 1<sup>+</sup>*Ly* 23<sup>-</sup> are *Ly* 1 cells and *Ly* 1<sup>-</sup>*Ly* 23<sup>+</sup> cells are *Ly* 23 cells.

tested C57BL cytotoxic T cells generated against alloantigens. These authors observed a substantial (58%) reduction in cytotoxicity after the elimination of *Ly* 1 cells. The cytotoxic activity of secondary effector T cells sensitized *in vitro* to ectromelia virus-infected cells is significantly reduced after lysis of *Ly* 2 cells (Pang *et al.*, 1976). It is of interest that primary ectromelia immune cytotoxic T cells generated *in vivo* seem to be richer in *Ly* 1 cells than secondary cells.

Recent findings indicate that *Ly* 1 and *Ly* 23 cells are stable and apparently do not give rise to one another. These results imply that the two sub-populations represent the products of distinct lines of differentiation. Experiments have been designed to determine whether *Ly* 1 cells are programmed for helper activity regardless of external conditions. Eardley *et al.*, (1978) found that *in vitro* stimulation of purified populations of *Ly* 1 cells under culture conditions devised to induce optimal suppressive activity, invariably results in the generation of T helper but not of T suppressor activity. These results support the view that the genetic program for a single differentiated set of cells combines information for surface phenotype and function.



## CHAPTER 2

### INTRODUCTION

T cells play an essential role in the process of recovery from primary infection of mice with ectromelia virus, a poxvirus related to vaccinia and variola viruses (Blanden, 1970, 1971a, 1971b; Blanden et al., 1975a) but the precise mechanisms involved, and the participation of different effector T cell subsets and ancillary cells remain to be fully elucidated. Massive liver necrosis is the major cause of death (Blanden, 1970, 1971a, 1971b). Recovery therefore depends upon control of virus replication and spread in progressing lesions of the liver parenchyma, and upon control of infection in other tissues, such as spleen, which may contribute to the cell-associated viremia (Blanden, 1970) that in turn may initiate further liver lesions.

### A SINGLE GENETIC ELEMENT IN *H-2K* AFFECTS MOUSE T CELL ANTIVIRAL FUNCTION IN POXVIRUS INFECTION

A number of potential mechanisms of recovery could be supplied by effector T cells. First, virus-infected cells are killed before virus replication and spread (Blanden et al., 1975b). This is feasible since ectromelia virus is highly cytopathic and is eliminated by cytotoxic T cells long before progeny virus is assembled (Blanden et al., 1976). Second, recognition of foci of infection by effector T cells leads to recruitment (Blanden, 1971a, 1971b, 1971c and others; Blanden and Ming, 1973) of nonnuclear phagocytes which destroy virus-infected material (Blanden, 1971b). Third, effector T cells may secrete lymphokines at sites of infection, thus protecting other cells as the immediate vicinity (Blanden, 1971b; Glasgow, 1970). Available evidence indicates an important role for helper T cells and antibody in the normal process of recovery (Blanden, 1970, 1971a, 1971b; Blanden et al., 1975a).

All of the mechanisms described above would require specific recognition of virus-induced antigenic patterns, since the protection conferred by immune T cells is virus-specific (Blanden, 1971a). However,

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A number of potential mechanisms of recovery could be supplied by effector T cells. First, cytotoxic T cells could kill virus-infected cells before virus replication is completed, thus halting virus spread; this is feasible since ectromelia-infected cells display antigenic changes recognized by cytotoxic T cells long before progeny virus is assembled (Ada *et al.*, 1976). Second, recognition of foci of infection by effector T cells leads to recruitment (Blanden, 1971a, 1971b, 1974) and activation (Blanden and Mims, 1973) of mononuclear phagocytes which destroy virus-infected material (Blanden, 1971b). Third, effector T cells may secrete interferon at sites of infection, thus protecting other cells in the immediate vicinity (Blanden, 1971b; Glasgow, 1970). Available evidence denies an important role for helper T cells and antibody in the normal process of recovery (Blanden, 1970, 1971a, 1971b; Blanden *et al.*, 1975a).

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recent findings in a number of different systems indicate that cytotoxic T cells do not simply recognize virus-specified antigenic determinants, but that host cell gene(s) in the *H-2* gene complex are involved in producing new antigenic patterns on infected cells (Zinkernagel and Doherty, 1974; Shearer, 1974; Gardner *et al.*, 1975; Bevan, 1975; Gordon *et al.*, 1975; Doherty and Zinkernagel, 1975).

## MATERIALS AND METHODS

### Animals

All mouse strains were bred at the John Curtin School and used at 7-10 wks of age.

### Virus strains

Ectromelia virus stocks of both attenuated (Hampstead egg) and virulent (Moscow) strains were used. Virulent Moscow strain ectromelia virus was obtained in the form of 0.95% saline suspension from homogenates of infected mouse spleens. The homogenates were dispersed by ultrasonication and centrifuged to remove debris.

Stocks of attenuated Hampstead egg strain ectromelia virus were obtained from infected chorioallantoic membranes of 14-day old chick embryos. All virus stocks were stored in small portions at  $-70^{\circ}\text{C}$ .

### Titration of ectromelia virus

The method used was based on that described previously (Blanden, 1970). L-929 cells were seeded into 35 mm wells of plastic tissue culture trays (Linbro Chemical Co., New Haven, Conn., U.S.A.) at a density of  $10^6$  cells/well in 5 ml of a modified medium consisting of Eagle's minimum essential medium (MEM) containing 5% heat-inactivated ( $56^{\circ}\text{C}$  for 60 minutes) bovine serum and 2% heat-inactivated foetal calf serum (FCS). Confluent monolayers were infected with virus suspended in MEM containing 1% FCS using 0.1 ml per

dish for 60 minutes at 37°C, and then overlaid with 5 ml modified medium containing 0.5% w/v carboxymethyl cellulose (low viscosity sodium salt, BDH Chemicals, Poole, England). Infected cultures were incubated at 35°C for 5 days, overlay medium removed and wells stained with crystal violet (5 gm crystal violet, 500 ml phosphate-buffered saline, 2 ml 70% glutaraldehyde). Amounts of virus are expressed in plaque-forming units (PFU).

#### Preparation of cell suspensions for transfer

Spleens were cut into pieces and pressed through stainless steel sieves into Puck's A saline (0.8% NaCl, 0.04% KCl, 0.1% glucose, 0.035% NaHCO<sub>3</sub>, 0.002% phenol red in deionized water). After dissociation of cell clumps by pipetting, capsular and fibrous material was deposited by increasing the pH to approximately 8 and centrifuging gently for 30 seconds; the cells remained in the supernatant. After washing three times, the cells were suspended for intravenous injection in Puck's A saline at pH 6, since at this pH embolic effects were minimized.

#### Plan of cell transfer experiments

Donors of immune spleen cells were immunized intravenously (i.v.) with  $2 \times 10^4$  PFU of attenuated virus and their spleens harvested 5 days later at about the peak of the effector T cell response (Blanden, 1971a; Blanden and Gardner, 1976). Cell suspensions were transferred i.v. to recipients which had been infected i.v. 24 hours previously with virulent virus. Transferred antiviral effects were determined by titrating virus in individual spleens (and sometimes livers) of groups of four to five recipients sacrificed 24 hours after cell transfer. Data from spleen and liver are essentially similar (Blanden, 1971a) but spleens are more conveniently titrated, and spleen data only are given



here. The collected spleens were stored at  $-70^{\circ}\text{C}$  until titration.

#### Preparation of spleens for virus titration

The spleens were homogenized in 2 ml MEM with a motorized teflon pestle, and the homogenate dispersed by ultrasonication and centrifuged to remove debris. Portions of the supernatant were then diluted into MEM and serial 10-fold dilutions were made. The dilutions were then titrated for virus content as described above.

#### Cell culture

Continuous line mouse fibroblasts (L-929) were grown in MEM with 10% bovine serum and 100  $\mu\text{g/ml}$  of penicillin and streptomycin, and incubated at  $37^{\circ}\text{C}$  in sealed tissue culture flasks (Falcon Plastics).

#### Statistical methods

Means and standard errors of the means (SE) were calculated from groups of four to five mice. Significance was determined by Student's *t* test.

#### RESULTS AND DISCUSSION

Transfer of antiviral activity occurred only in donor-recipient combinations which shared either *K* or *D* region genes (Table 1). *I* region homology was neither sufficient nor necessary and the remainder of the genotype seemed irrelevant. These results thus conform with previous data indicating a requirement for *K* or *D* region genes in expression of T cell-mediated lysis of virus-infected or TNP-modified target cells *in vitro* (Blanden *et al.*, 1975b; Shearer *et al.*, 1975). Cytotoxic T cells may therefore play a central role in recovery, particularly since the kinetics of production in the spleen of T cells with *in vivo* antiviral function (as described here) and *in vitro* cytotoxicity are similar (Blanden and Gardner, 1976). However, this evidence is indirect, and a firm conclusion cannot yet be drawn.

Since *I* region homology between donors and recipients is sufficient for other T cell functions such as helper activity (Katz *et al.*, 1975) and delayed-type hypersensitivity (DTH) to non-viral antigens (Miller *et al.*, 1975), the present data indicate that the effector T cells responsible for virus clearance, whatever the mechanisms they employ or trigger, are a subset which is defined by the requirement for operation of gene(s) in the *K* or *D* regions of the *H-2* complex.

The molecular basis for the phenomena cited above poses important questions. Let us assume that immunocompetent, precursor T cells employ the same mechanisms of antigen recognition while interacting with infected "stimulator" cells during the process of induction of the effector T cell response, as employed by their progeny (effector T cells) which lyse infected target cells *in vitro*, or trigger virus clearance *in vivo*. Potential models for these interactions have been proposed and tested by Zinkernagel and Doherty (1975a). Available evidence from experiments with lymphocytic choriomeningitis (LCM) virus (Zinkernagel and Doherty, 1975a, 1975b), ectromelia virus, trinitrophenyl (TNP)-modified cells (Shearer *et al.*, 1975) and minor histocompatibility antigens (Bevan, 1975) supports the following model. T cells interact with stimulator or target cells *via* receptors for antigen only, but the major antigenic patterns recognized are not simply virus-specified; they incorporate features dictated by *K* or *D* region genes. This raises questions concerning the way in which *K* or *D* region genes and/or their products interact with the viral genome, or its products, or other antigens such as TNP, to produce new antigenic patterns which stimulate precursors of effector T cells. It also provokes speculation as to the nature of T cell receptors for antigen, and the scope of receptor 'dictionaries' on various T cell subsets.



Further characterization of the nature of the genes in *H-2K* or *H-2D* regions is relevant to these questions. We therefore investigated B6.C-*H-2*<sup>ba</sup> mice, which bear a mutation which arose in the *K* end of the *H-2* complex (Bailey *et al.*, 1971) and has now been extensively characterized. F<sub>1</sub> hybrids of B6.C-*H-2*<sup>ba</sup> and another mutant of C57BL/6 reject C57BL/6 skin grafts (Apt *et al.*, 1975), thus suggesting strongly that each mutation involved the same single genetic element. Since C57BL/6 and B6.C-*H-2*<sup>ba</sup> mice also give reciprocal mixed lymphocyte reactions (MLR), cell-mediated lympholysis (CML), and graft-versus-host reactions (GVHR), it seems that T cells recognize the antigenic pattern(s) affected by the mutation, and that *K* region was the site of change (Nabholz *et al.*, 1975; Forman and Klein, 1975). However, the structure of antigenic determinants recognized by B cells appear qualitatively unchanged by the mutation on the basis of sound serological evidence (Bailey *et al.*, 1971; Klein *et al.*, 1974).

The data in Table 1 clearly show the effect of the B6.C-*H-2*<sup>ba</sup> mutation on T cell antiviral function *in vivo*. Immune T cells from B10.A(5R) mice, which reduced virus titres very efficiently in C57BL/6 mice, because of shared *K* region, did not have a significant protective effect in B6.C-*H-2*<sup>ba</sup> recipients, whereas T cells from B10.A(2R) mice operated efficiently in B6.C-*H-2*<sup>ba</sup> recipients because of *D* region homology. These results therefore suggest that the antigenic patterns induced by ectromelia infection which are recognized by effector T cells do not directly involve serologically defined *H-2* antigenic determinants. Instead, they seem to be controlled partly by a single genetic element responsible for alloantigenic patterns recognized by T cells in such reactions as MLR, CML, GVHR and graft rejection (Nabholz *et al.*, 1975; Forman and Klein, 1975). What is the nature of this genetic

element? Is it a single cistron coding for a polypeptide, as the conventional view of the complementation results would suggest (Apt *et al.*, 1975)? Or is it a more complex genetic unit? Further characterization of the virus-induced antigenic patterns recognized by effector T cells may clarify this issue.

Finally, in view of the large quantitative effects of *K* or *D* region genes reported here, the rational exploitation in clinical medicine of T cell-mediated mechanisms against infections and tumors, perhaps including transfer factor (Lawrence, 1972), could well be optimized by investigation of genetic regions analogous to *K*, *D*, and *I* in the HLA complex.

#### SUMMARY

Cell transfer experiments using mice with recombinant *H-2* haplotypes were used to map the *H-2* regions which must be shared by ectromelia-immune T cell donors and virus-infected recipients for transfer of virus clearance mechanisms in the spleen. *K* or *D* region genes were necessary and sufficient; *I* region genes were not involved. The remainder of the mouse genome could be varied widely without impairing the efficacy of T cell antiviral function, provided either a *K* or a *D* region was shared in the donor-recipient combination. A mutation in a single genetic element of the *K* region of the *H-2* complex abolished the antiviral effect of immune T cell transfer in a donor-recipient combination which shared the *K* end.



## REFERENCES

- Ada, G. L., Jackson, D. C., Blanden, R. V., Tha Hla, R. and Bown, N. A. 1976. Changes in the surface of virus-infected cells recognised by cytotoxic T cells. I. Minimal requirements for lysis of ectromelia-infected P-815 cells. *Scand.J.Immunol.* 5:23.
- Apt, A. S., Blandova, Z., Dishkant, I., Shumova, T., Vedernikov, A. A. and Egorov I. K. 1975. Study of H-2 mutations in mice. IV. A comparison of the mutants M505 and H21 by skin grafting and serological techniques. *Immunogenetics* 1:444.
- Bailey, D. W., Snell, G. D. and Cherry, M. 1971. Complementation and serological analysis of an H-2 mutant, in *Immunogenetics of the H-2 system*, A. Lengerova and M. Vajtiskova, eds. Karger, Basel, p. 155.
- Bevan, M. J. 1975. Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. *Nature (London)* 256:419.
- Blanden, R. V. 1970. Mechanisms of recovery from a generalized viral infection : Mousepox. I. The effects of anti-thymocyte serum. *J.Exp.Med.* 132:1035.
- Blanden, R. V. 1971a. Mechanisms of recovery from a generalized viral infection : Mousepox. II. Passive transfer of recovery mechanisms with immune lymphoid cells. *J.Exp.Med.* 133:1074.
- Blanden, R. V. 1971b. Mechanisms of recovery from a generalized viral infection : Mousepox. III. Regression of infectious foci. *J.Exp.Med.* 133:1090.
- Blanden, R. V. and Mims, C. A. 1973. Macrophage activation in mice infected with ectromelia or lymphocytic choriomeningitis virus. *Aust.J.Exp.Biol.Med.Sci.* 51:393.
- Blanden, R. V. 1974. T cell response to viral and bacterial infection. *Transplant.Rev.* 19:56.
- Blanden, R. V., Bown, N. A., Pang, T. E., Gardner, I. D. and Parish, C. R. 1975a. Effects of thymus-independent (B) cells and the H-2 gene complex on antiviral function of immune thymus-derived (T) cells. *Aust.J.Exp.Biol.Med.Sci.* 53:187.
- Blanden, R. V., Doherty, P. C., Dunlop, M. B. C., Gardner, I. D., Zinkernagel, R. M. and David, C. S. 1975b. Genes required for cytotoxicity against virus-infected target cells in K and D regions of the H-2 complex. *Nature(London)* 254:269.
- Blanden, R. V. and Gardner, I. D. 1976. The cell-mediated immune response to ectromelia virus infection. I. Kinetics and characteristics of the primary effector T cell response *in vivo*. *Cell.Immunol.* 22:271.

- Doherty, P. C. and Zinkernagel, R. M. 1975. A biological role for the major histocompatibility antigens. *Lancet* p. 1406.
- Forman, J. and Klein, J. 1975. Analysis of *H-2* mutants : evidence for multiple CMI target specificities controlled by the *H-2K<sup>b</sup>* gene. *Immunogenetics* 1:469.
- Gardner, I. D., Bower, N. A. and Blanden, R. V. 1975. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. III. Role of the *H-2* gene complex. *Eur.J.Immunol.* 5:122.
- Glasgow, L. A. 1970. Cellular immunity in host resistance to viral infections. *Arch.Intern.Med.* 126:125.
- Gordon, R. D., Simpson, E. and Samelson, L. E. 1975. *In vitro* cell-mediated immune responses to the male specific (H-Y) antigen in mice. *J.Exp.Med.* 142:1108.
- Katz, D. H., Graves, M., Dorf, M. E., Dimuzio, H. and Benacerraf, B. 1975. Cell interactions between histoincompatible T and B lymphocytes. VII. Cooperative responses between lymphocytes are controlled by genes in the *I* region of the *H-2* complex. *J.Exp.Med.* 141:263.
- Klein, J., Hauptfeld, M. and Hauptfeld, V. 1974. Serological distinction of mutants B6.C-H(21) and B6.M505 from strain C57BL/6. *J.Exp.Med.* 140:1127.
- Lawrence, H. S. 1972. Immunotherapy with transfer factor. *New Engl.J.Med.* 287:1092.
- Miller, J. F. A. P., Vadas, M. A., Whitelaw, A. and Gamble, J. 1975. *H-2* gene complex restricts transfer of delayed-type hypersensitivity in mice. *Proc.Natl.Acad.Sci. USA* 72:5095.
- Nabholz, M., Young, H., Meo, T., Miggiano, V., Rijnbeck, A. and Shreffler, D. 1975. Genetic analysis of an *H-2* mutant, B6.C-H-2<sup>b</sup>, using cell-mediated lympholysis: T and B cell dictionaries for histocompatibility determinants are different. *Immunogenetics* 1:457.
- Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur.J.Immunol.* 4:527.
- Shearer, G. M., Rehn, T. G. and Garbarino, C. A. 1975. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the *H-2K* and *H-2D* serological regions of the murine major histocompatibility complex. *J.Exp.Med.* 141:1348.
- Zinkernagel, R. M. and Doherty, P. C. 1974. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature(London)* 248:701.



Zinkernagel, R. M. and Doherty, P. C. 1975a. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature(London)* 251:547.

Zinkernagel, R. M. and Doherty, P. C. 1975b. H-2 compatibility requirement for T cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities are associated with structures coded for in H-2K or H-2D. *J.Exp.Med.* 141:1427.

- a Experimental procedure is described in MATERIALS AND METHODS. All donors were immunized i.v. with  $2 \times 10^4$  PFU of attenuated virus. Recipients were injected i.v. with  $2 \times 10^4$  of virulent virus for experiments 1 and 2, and with  $5 \times 10^5$  PFU for experiment 3. Viable immune spleen cell doses, given i.v. to each recipient were  $5 \times 10^7$ ,  $7 \times 10^7$  and  $15 \times 10^7$  for experiments 1, 2 and 3 respectively.
- b H-2 maps are for *K*, *I-A*, *I-B*, *I-C*, *S*, and *D* regions of the gene complex. *K* region of B6.C-H-2<sup>ba</sup> is designated "ba" on the basis of evidence cited in the text.
- c Results are expressed as mean  $\log_{10}$  virus PFU per organ  $\pm$  SE in groups of 4 mice. The limit of detection was 20 PFU/spleen ( $\log_{10} = 1.30$ ).
- d Significantly less than control groups given no cells ( $P < 0.01$ ).



TABLE 1

H-2 REQUIREMENT FOR TRANSFER<sup>a</sup> OF ANTIVIRAL EFFECT BY ECTROMELIA-IMMUNE T CELLS

Experiment	Immune T cell donors		Virus titres in recipient spleens 24 hours after cell transfer		
1	Strain	H-2 map <sup>b</sup>	Strain	H-2 map <sup>b</sup>	Titre <sup>c</sup>
	BALB/c	dddddd	BALB/c	dddddd	< 1.3 <sup>d</sup>
	C3H.OH	dddjdk	BALB/c	dddddd	< 1.3 <sup>d</sup>
	D2.GD	ddbbbb	BALB/c	dddddd	< 1.3 <sup>d</sup>
	B10.A(2R)	kkkddb	BALB/c	dddddd	5.1 ± 0.1
	A.TFR 2	ffffsd	BALB/c	dddddd	1.5 ± 0.2 <sup>d</sup>
	Nil		BALB/c	dddddd	5.1 ± 0.1
2	CBA/H	kkkkkk	A.TL	skkkkd	4.6 ± 0.4
	SJL/J	ssssss	A.TL	skkkkd	< 1.3 <sup>d</sup>
	BALB/c	dddddd	A.TL	skkkkd	< 1.3 <sup>d</sup>
	Nil		A.TL	skkkkd	4.9 ± 0.9
	CBA/H	kkkkkk	CBA/H	kkkkkk	< 1.3 <sup>d</sup>
	Nil		CBA/H	kkkkkk	5.1 ± 0.3
3	B6.C-H-2 <sup>ba</sup>	"ba"bbbb	B6.C-H-2 <sup>ba</sup>	"ba"bbbb	3.3 ± 0.6 <sup>d</sup>
	B10.A(5R)	bbdddd	B6.C-H-2 <sup>ba</sup>	"ba"bbbb	6.0 ± 0.2
	B10.A(2R)	kkkddb	B6.C-H-2 <sup>ba</sup>	"ba"bbbb	< 1.3 <sup>d</sup>
	Nil		B6.C-H-2 <sup>ba</sup>	"ba"bbbb	6.5 ± 0.1
	B10.A(5R)	bbdddd	C57BL/6	bbbbbb	< 1.3 <sup>d</sup>
	Nil		C57BL/6	bbbbbb	5.5 ± 0.2

## CHAPTER 3

### INTRODUCTION

It has been shown that T cells are essential in the process of recovery from primary infection of mice with ectromelia virus, a natural mouse pathogen (Blanden, 1970, 1971a, 1971b; Blanden et al., 1971a). T cells harvested from spleens at the peak of the primary response after i.v. immunization of donors with attenuated virus cause highly significant reductions in virus titres in target organs (e.g., liver and spleen) of recipient, syngeneic animals (Zinkernagel et al., 1975). Using mice with recombinant H-2 haplotypes, it could be demonstrated that T cell donors and virus-infected recipients must share H-2K or H-2D region genes for transfer of virus clearance to be effective (see also Blanden, 1976; see Chapter 2). This and other evidence (Blanden and Gieding, 1976) suggests that T cells which are cytotoxic for virus-infected cells and which release cytotoxic factors are the effector cells.

### PROTECTIVE ACTIVITY OF SECONDARY EFFECTOR T CELLS

#### GENERATED *IN VITRO* AGAINST ECTROMELIA VIRUS

#### INFECTION *IN VIVO*

Procedures are to be fully described in a separate paper. The similarity or otherwise between H-2 restriction in mice, and the role of HLA antigens in man would need to be investigated. Also, in vitro methods to generate protective T cells would be an advantage since they would not require human cell donors to undergo the risk and inconvenience of immunization with virus. In this report, we describe investigations into the latter of these two problems, viz. the antiviral effects *in vivo* of effector T cells generated *in vitro*.

### MATERIALS AND METHODS

#### Mice

Inbred mice were bred at the John Curtin School and used at 4-6 weeks of age. In any one experiment, mice of the same sex were used.



## INTRODUCTION

It has been shown that T cells are essential in the process of recovery from primary infection of mice with ectromelia virus, a natural mouse pathogen (Blanden, 1970, 1971a, 1971b; Blanden, *et al.*, 1975a). T cells harvested from spleens at the peak of the primary response after i.v. immunization of donors with attenuated virus cause highly significant reductions in virus titres in target organs (e.g., liver and spleen) of recipient, syngeneic animals (Zinkernagel *et al.*, 1975). Using mice with recombinant H-2 haplotypes, it could be demonstrated that T cell donors and virus-infected recipients must share H-2K or H-2D region genes for transfer of virus clearance to be effective (Kees and Blanden, 1976; see Chapter 2). This and other evidence (Blanden and Gardner, 1976) suggests that T cells which are cytotoxic for virus-infected cells and which recognize H-2K or H-2D antigens plus virus-specific antigens trigger the process of viral clearance *in vivo*. If cell transfer procedures are to be fully exploited in clinical medicine, the similarity or otherwise between H-2 restriction in mice, and the role of HLA antigens in man would need to be investigated. Also, *in vitro* methods to generate protective T cells would be an advantage, since they would not require human cell donors to undergo the risk and inconvenience of immunization with virus. In this report, we describe investigations into the latter of these two problems, *viz.* the antiviral effects *in vivo* of effector T cells generated *in vitro*.

## MATERIALS AND METHODS

### Mice

Inbred mice were bred at the John Curtin School and used at 6-8 wks of age. In any one experiment, mice of the same sex were used.

CBA/H, C57BL/6 and BALB/c strains were bred and reared under specific pathogen-free conditions, whilst A.TL were bred and reared under conventional conditions. Unless stated otherwise, CBA/H mice were used.

### Cell preparation

The methods used for cell preparation have been described in Chapter 2.

### Viruses

Ectromelia virus stocks of both virulent (Moscow) and attenuated (Hampstead egg) strains were used. The methods of growing and titrating these strains have been described in Chapter 2. Amounts of virus are expressed as plaque-forming units (PFU).  $\gamma$ -irradiated virus was subjected to  $10^6$  Rads from a  $^{60}\text{Co}$  source. In any one experiment, recipients of immune cells were given an i.v. virus dose which resulted in a splenic titre of approximately  $10^5$ - $10^6$  PFU 48 hours after injection.

The stocks of Armstrong and WE3 strain LCM viruses used were from guinea pig lung or spleen. The methods for the preparation of virus stocks were the same as for ectromelia virus, described in Chapter 2. The plaque assay for LCM virus was the same as for ectromelia virus except that virus dilutions were made in 0.5% gelatine saline and the plates were incubated for 6 to 7 days (Dunlop, 1978).

### Immunization

Mice were infected i.v. with  $2 \times 10^4$  PFU of Hampstead egg strain ectromelia virus. Spleens were removed 5 days after infection at about the peak of the primary effector T cell response, or if used as a source of memory responder cells, spleens were taken after 4-6 weeks. Priming dose for LCM virus was 2500

PFU WE3 or 8000 PFU Armstrong LCM virus.

#### Memory cell culture using ectromelia virus

The method used was essentially similar to that described previously (Gardner and Blanden, 1976). The procedure used was: Spleen cells harvested from mice previously immunized with attenuated ectromelia virus (responder cells) were cultured with syngeneic spleen cells infected with  $\gamma$ -irradiated virulent ectromelia virus (stimulator cells). Unless otherwise stated CBA/H cells were used. Stimulator cells were infected with 1-2 PFU/cell and a stimulator to responder ratio of 1:5 was used. Cultures were set up in tissue culture flasks (Falcon Plastics, 25 cm<sup>2</sup> or 75 cm<sup>2</sup> growth area) at a final concentration of  $2 \times 10^6$ /ml in 10 ml/25 cm<sup>2</sup> flask or 40 ml/75 cm<sup>2</sup> flask and incubated at 39°C (a non-permissive temperature for ectromelia replication which prevents the virus from exerting cytopathic effects against responder cells). The complete culture medium was Eagle's minimal essential medium with non-essential amino acids (Grand Island Biological Co., Grand Island, New York, USA, catalogue No. F-15) with 10% foetal calf serum (FCS),  $10^{-4}$  M 2-mercaptoethanol and antibiotics. Cultures were harvested after 5 days and dead cells were removed (see below) before transfer to recipient mice or cytotoxicity assay *in vitro*.

#### Lymphocytic choriomeningitis (LCM) virus memory culture

The method used was similar to that for ectromelia virus except that macrophages from the peritoneal cavity were used as stimulators and incubation was at 37°C for 5 days. The cultures were made by Dr. M. B. C. Dunlop and methods have been fully described by Dunlop and Blanden (1976).

#### Removal of dead cells

After harvesting of responder cells from secondary responses,



dead cells were removed by centrifuging through Isopaque/Ficoll as described in detail by Davidson and Parish (1975). A cell suspension containing from  $10^7$  to  $10^8$  lymphocytes in 5 ml medium (F-15 or tissue culture medium) containing 10% FCS was brought to  $20^{\circ}\text{C}$ . This preparation was layered gently onto 4 ml of separating medium which had also been prewarmed to  $20^{\circ}\text{C}$  and which consisted of 12 parts of 14% (w/v) Ficoll (Pharmacia, Uppsala, Sweden) dissolved in distilled water and 5 parts of 32.8% (w/v) sodium metrizoate (Isopaque; Nyegaard and Co., Oslo, Norway), the complete mixture containing 0.1% (w/v) sodium azide. The separations were carried out in 12 ml U-bottomed, polycarbonate centrifuge tubes (Ivan Sorvall Inc., Norwalk, Conn., USA). The tubes were spun at  $20^{\circ}\text{C}$  at 2000 g for 15 minutes. The white cell layer at the interface was collected and washed twice with medium containing 10% FCS.

#### Radio-isotope labelling of cells

$10^8$  cells were suspended in 1 ml F-15 containing 1% FCS and 50  $\mu\text{Ci}$   $^{51}\text{Cr}$  (sodium chromate, specific activity 100 - 400 mCi/mg of chromium, C.A.E., Gif sur Yvette, France). Cells were incubated for 30 minutes at  $37^{\circ}\text{C}$  and washed three times with F-15 containing 10% FCS.

#### Cytotoxicity assay using L929 cells

L929 cells were incubated at  $37^{\circ}\text{C}$  for 60 minutes in serum-free medium at a concentration of 100 - 150  $\mu\text{Ci}$  of  $^{51}\text{Cr}/10^6$  cells. After two washings with F-15 containing 10% FCS a portion of the cells was infected with Moscow strain ectromelia virus at a density of  $10^7$  cells/ml F-15 with 4 - 10 PFU/cell. The cells were incubated at  $37^{\circ}\text{C}$  for 60 minutes and washed twice with F-15 containing 10% FCS. (For LCMV infection, L929 cells were infected with neat WE3-LCM virus stock

20 hours prior to assay.)  $2 \times 10^4$  cells were distributed into wells of 96-well (6 mm diameter) plastic tissue culture trays (Linbro Chemical Co., New Haven, Conn., USA) in 0,1 ml of F-15 containing 10% FCS with an automatic pipette. Two hours later graded numbers of immune spleen cells were added in 0,1 ml of the same medium. Plates were incubated at  $37^\circ\text{C}$  in a 10%  $\text{CO}_2$ , 7%  $\text{O}_2$ , 83%  $\text{N}_2$  atmosphere for 6 hours. After the incubation 0,1 ml of the supernatant was removed from each well and the  $\gamma$ -emission in the supernatant was counted in a Packard Auto-Gamma spectrometer. Total releasable radioactivity from the target cells (water lysis) was obtained by adding 0,1 ml of labelled cells ( $2 \times 10^4$  cells) to 0,9 ml of distilled water. These tubes were incubated in parallel with the assay, then centrifuged and the supernatant and the pellet counted to calculate the percentage of  $^{51}\text{Cr}$  releasable by water, (usually 80 to 90% of the total).

Lysis in test wells (expressed as a percentage of the lysis achieved by water) was obtained from the formula:

$$\frac{\text{counts in 0,1 ml supernatant from test wells} \times 2 \times 100}{\text{mean counts in supernatant of water lysis tubes (triplicates)}}$$

Spontaneous  $^{51}\text{Cr}$  release, i.e., the release from the target cells incubated with medium only, was calculated similarly (usually at between 7 and 12%). The mean (calculated from triplicates) of the spontaneous release was subtracted from the lysis in test wells to give the specific  $^{51}\text{Cr}$  release. All results show the mean percentage of specific  $^{51}\text{Cr}$  release in triplicate assay wells. Standard errors of the means were less than 2.4% and are omitted from the tables for clarity.

#### Cytotoxicity assay using macrophage target cells

Macrophages were collected by washing out the peritoneal cavity



of mice with 5 ml ice-cold Puck's A saline,  $10^5$  cells were distributed into 6 mm wells of 96-well plastic tissue culture trays (see above) in 0,2 ml F-15 containing 10% FCS and left for 16 to 18 hours. Conditions for the incubation of plates are given above. The cells were then washed three times to remove nonadherent cells and labelled with 2  $\mu$ Ci  $^{51}\text{Cr}$  in 0,05 ml of F-15/well for 60 minutes at  $37^\circ\text{C}$ . After three washes with F-15 containing 10% FCS the macrophages were infected with Moscow strain ectromelia virus (grown in eggs) for 60 minutes at  $37^\circ\text{C}$  (10 PFU/cell in 20  $\mu$ l F-15). Cells were then washed and left for 3 - 4 hours at  $37^\circ\text{C}$ , and the immune cells added in a total volume of 0,2 ml medium containing 5% FCS. Plates were incubated as described above. The supernatant was carefully pipetted off. Cells remaining in the wells were incubated at  $37^\circ\text{C}$  for 1 hour with 0,3 ml of distilled water, mixed vigorously and these cell lysates and the supernatants were counted as described above.

Total releasable radioactivity from the target cells (water lysis) was obtained as described above, except that only 0,3 ml of distilled water was added. The following formula was used to calculate the percentage of  $^{51}\text{Cr}$  released in the test wells:

$$\frac{\text{counts in supernatants} \times 100}{\text{counts in supernatants} + \text{counts in cell lysates}} \times \frac{100}{\% \text{ water lysis}}$$

Spontaneous  $^{51}\text{Cr}$  release (for target cells incubated with medium only) was similarly calculated and the mean of triplicate wells subtracted from the lysis in test wells to give the specific  $^{51}\text{Cr}$  release. Results are expressed as the mean percentage of specific  $^{51}\text{Cr}$  release in triplicate assay wells. Standard errors of the means were less than 3% and are omitted from the tables for clarity.

#### Treatment with anti-theta ascitic fluid and complement

Anti-theta ascitic fluid was obtained from AKR/J mice injected with thymocytes from CBA/H mice. Ascitic fluid was collected and tested for specificity against CBA/H T cells as described by Blanden and Langman (1972). For the treatment, cells were suspended at  $2 \times 10^7$  viable cells/ml in a 1:9 dilution of anti-theta ascitic fluid in F-15 and incubated at room temperature for 30 minutes. After washing twice, the cells were resuspended in a 1:3 dilution of absorbed rabbit complement in F-15 and incubated at  $37^\circ\text{C}$  for 30 minutes. (Selected rabbit sera were used and were absorbed prior to use with spleen cells from outbred mice and from mice of the strain used in the experiment until all nonspecific activity was removed.) The cell viability was determined by Trypan Blue exclusion.

#### Statistical methods

Significance was determined by Student's t test.

### RESULTS

#### Protection by secondary effectors given 24 hours after virus infection

It has been established (Blanden, 1971a) that an adequate procedure to measure protection *in vivo* mediated by transferred immune cells is to determine the virus titre reduction in spleen and liver of infected recipients 24 hours after cell transfer. Since a dose of  $1.4 \times 10^7$  effector cells from the peak of the primary T cell response in the spleen *in vivo* effectively reduces virus titres in spleens of infected recipients (Zinkernagel *et al.*, 1975), a similar dose of secondary effector cells generated *in vitro* was first tested for antiviral activity. Groups of four CBA/H mice were injected i.v. with  $2.5 \times 10^6$  PFU of virulent virus and injected i.v. 24 hours later with  $1.5 \times 10^7$  secondary effector cells. Recipients were killed 24 or 48 hours after cell transfer and spleens were titrated for virus. This cell dose did not reduce virus titres significantly (Table 1). Up to  $8 \times 10^7$  cells



were required to show a significant protective effect *in vivo* (Table 1), despite repeated previous observations that the potency of secondary cells was at least 8-fold greater than primary T cell populations in the cytotoxicity assay *in vitro* (Blanden *et al.*, 1977).

These results could mean that T cells which are cytotoxic for infected targets *in vitro* are not those responsible for virus clearance *in vivo*. However, another possibility is that the post-injection migration of five day primary immune cells generated *in vivo* is different from that of secondary effectors generated *in vitro*, perhaps as a consequence of cell surface changes induced *in vitro* in the latter case. Studies with  $^{51}\text{Cr}$  labelled cell populations of both types were carried out. Since 60-70% of the counts were present in the lung, liver and spleen 1 hour after i.v. injection, the sum of counts in these organs was designated as 100%, and relative proportions of the sum present in individual organs at various times after injection were calculated. One hour after injection, 47% of secondary cell counts were in the lungs, with 33% in liver and 20% in spleen. In contrast, in the case of primary cells, only 30% of counts were in the lungs, with 43% in liver and 27% in spleen. These differences in cell localization were highly significant ( $P < 0.02$  in groups of 3 mice). At 8 and 24 hours after injection, the differences between primary and secondary cell localization became less significant as the cells apparently migrated from the lungs to the liver and spleen and as label was spontaneously released from the cells. For example, at 24 hours, 7% of secondary cell counts were in the lungs, with 54% in liver and 39% in spleen; at this time, 4% of primary cell counts were in the lungs, with 61% in liver and 35% in spleen. These findings are consistent with the possibility that after i.v. injection the relatively weak antiviral effects of secondary cells in terms of

spleen titre reduction are due to a protracted localization of effector cells in the lung, thus resulting in a time delay before they reach the spleen and exert their effects. However, it must be recognized that since virus-specific effector cells probably constitute a very small part of the total cell population (Gardner *et al.*, 1974), and since  $^{51}\text{Cr}$  is not a permanent cell label, this type of experimental approach cannot be considered definitive.

Preliminary attempts were made to prevent effector cells from being detained in lung capillaries by injecting them in medium containing Heparin (10 IU/ml) or by incubating them with CBA/H serum (10% in complete medium) for 4 hours prior to injection. Neither of these treatments improved the ability of the cells to reduce virus titres in spleens of recipients (data not shown). However, this problem could be of considerable practical importance in the future and will be pursued further.

Secondary effector cells transfer antiviral activity when virus is administered 24 hours after injection of cells

The preceding findings suggested that secondary cells may require more time than primary cells to exert antiviral effects in the spleen after i.v. injection because of differences in migration patterns. Further experiments explored this aspect. A dose of only  $2 \times 10^6$  secondary effector cells was effective in reducing virus titres in spleen when it was given simultaneously with, or 24 hours before, virus injection. In all cases, the recipients were killed 48 hours after injection of virus (Table 2).

In subsequent experiments the following order of administration was applied: Virus was given 24 hours after cell transfer and organs were harvested for virus titration 48 hours after injection of virus.



C57BL/6 mice were used to extend these observations to another strain. Table 3 shows that primary effector cells from the peak of the response *in vivo* (5 days after immunization) are as effective in reducing virus titres as a similar dose of secondary effectors, despite the higher potency of secondary cells in the cytotoxicity test *in vitro*.

#### Dose response relationship

Using the protocol in which virus was injected 24 hours after cells, and splenic titres in recipients were determined 48 hours after virus injection, the relationship between  $\log_{10}$  cell dose administered, and  $\log_{10}$  reduction in virus titres in recipients was investigated. With a fixed virus dose of  $2.5 \times 10^6$  PFU per mouse, cell doses lower than  $10^6$  were ineffective but with cell doses from  $10^6$  up to  $4 \times 10^6$ , the dose-response relationship appeared to be linear (Fig. 1).

#### Nature of cell transferring antiviral activity

Effector cells generated *in vivo* during infection with ectromelia virus are T cells (Blanden, 1971a; Gardner *et al.*, 1974). If these cells are used in a cell transfer experiment their antiviral activity is abrogated after treatment with anti-theta and complement (Blanden, 1971a). The secondary response *in vitro* also generates cytotoxic T cells (Gardner and Blanden, 1976). Since secondary effector cells treated with anti-theta and complement lost their antiviral activity (Table 4), they too appear to be T cells.

#### H-2 restriction of secondary T cells transferring antiviral activity

A.TL and CBA/H ( $H-2^k$ ) memory cells were used to generate secondary responses to syngeneic ectromelia-infected stimulators. The H-2 map for A.TL mice is skkkkd for the *K*, *I-A*, *I-B*, *I-C*, *S* and *D* regions respectively. The antiviral activity of A.TL effector cells was determined after transferring them to SJL ( $H-2^S$ ), BALB/c ( $H-2^d$ ) or CBA/H ( $H-2^k$ ) recipient mice

(Table 5). As a control, another group of CBA/H mice was given syngeneic effector cells to show that these mice can be protected with appropriate cells. A.TL effector cells reduced virus titres effectively in SJL (shared *K* region) and BALB/c mice (shared *D* region) but not in CBA/H recipients (shared *I-A*, *I-B*, *I-C*, *S* regions). This restriction of transferrable antiviral activity is similar to that observed with primary effector T cells generated *in vivo* (Kees and Blanden, 1976). Cytotoxic activity of secondary T cells against ectromelia-infected targets *in vitro* is similarly restricted (Pang and Blanden, 1977).

#### Virus specificity of protective effect of secondary T cells

Responder cells from mice primed with either ectromelia or LCM virus were cultured with stimulator cells infected with the same virus as used for primary stimulation. Effector cells from both cultures were tested for antiviral activity in groups of mice subsequently injected with either ectromelia or LCM virus. Cytotoxicity assays *in vitro* against infected target cells always show clear virus-specificity, i.e., only when donors of memory T cells, stimulator cells and target cells were all infected with the same virus did significant lysis occur (Table 6). In several cell transfer experiments however, significant protection of ectromelia-infected mice given secondary anti-LCM effector cells generated using the WE3 strain of LCM virus was observed. The converse did not occur, i.e., secondary anti-ectromelia effector cells reduced virus titres in ectromelia virus-infected mice, but not in LCM virus-infected mice (Table 7). A possible mechanism for the apparent one-way cross protection could be that infectious LCM virus was transferred together with anti-LCM effector cell populations and infected cells in the recipient. After injection with ectromelia virus these same cells could also become infected with



ectromelia and could express both types of virus-induced antigens on their surface membranes. They would then be lysed by anti-LCM secondary effectors. This could indirectly limit the multiplication of ectromelia virus.

In the following experiment, precautions were taken to minimize transfer of LCM virus with effector cells. Firstly, LCM virus-infected stimulator cells were fixed with formaldehyde to kill any infectious LCM virus present (Dunlop and Blanden, 1976). Secondly, the culture was set up with spleens from 'memory' mice primed more than 2 months previously with Armstrong LCM virus instead of the WE3 LCM virus strain used in previous experiments. Armstrong strain is known to persist for less than two months in infected animals (G. A. Cole and M. B. C. Dunlop, personal communication). Table 8 shows that bidirectional virus-specificity can be demonstrated under these conditions.

#### DISCUSSION

These experiments demonstrate that secondary effector cells generated *in vitro* against ectromelia virus infection mediate protection after transfer *in vivo*. The protective effect was measured as reduction of virus titres in spleens 48 hours after injection of virus and 72 hours after administration of the cells. If appropriate protocols are followed, bidirectional virus-specificity of the antiviral effect can be demonstrated in specificity tests carried out with secondary effectors generated against either ectromelia or LCM virus infection. A dose of  $2 \times 10^6$  cells is sufficient for significant virus titre reduction, provided the cells are administered 24 hours before virus injection.

There was, however, a discrepancy between the potency of effector cell populations displayed in the *in vivo* assay described above, and in

cytotoxicity against virus-infected target cells *in vitro*. In the former assay, potency of secondary cells generated *in vitro* was similar to that of primary cells generated *in vivo*; in the latter assay using the same cell populations, the secondary cells were at least four-fold more potent. Two explanations can be considered. Firstly, the T cells responsible for lysing infected target cells *in vitro* may not be solely responsible for triggering the antiviral effect *in vivo*. Secondly, the same cytotoxic T cells may be responsible for both lysis *in vitro* and viral clearance *in vivo*, but the migration patterns *in vivo* of cells generated in an *in vitro* system may prevent or delay full expression of their potential antiviral effects *in vivo*.

Though definitive proof is beyond experimental test, we favour the second explanation for the following reasons: evidence presented elsewhere shows that in the primary response *in vivo* the kinetics of generation of cytotoxic T cells and *in vivo* antiviral effectors is similar (Blanden and Gardner, 1976), and both require H-2K or H-2D region genes to be shared between donors of T cells and targets (or recipients) for effector function to be expressed (Blanden *et al.*, 1975b; Kees and Blanden, 1976). The antiviral effect *in vivo* mediated by secondary effector cells generated *in vitro* is also T cell-dependent, as shown in the present experiments by sensitivity to anti-theta treatment and requirement for H-2K or H-2D sharing by donor and recipient. Also, the present investigation did reveal a difference in organ localization pattern of intravenously injected cells, depending on whether they were obtained directly from donors, or were previously cultured *in vitro*; the latter cells showed a greater tendency to localize in the lungs and less localization in spleen and liver one hour after injection. Thus a possible explanation for the apparent lower activity of secondary effector cells *in vivo* is that cultured T cells



are detained in lung capillaries, and are therefore unable to trigger reduction of virus titres in the spleen for a considerable time after intravenous injection. This idea is also consistent with the finding that more efficient antiviral activity of transferred secondary T cells in the spleens of recipients was achieved by increasing the time interval between injection of the cells and the titration of virus in recipient spleens.

Development of methods to circumvent the problem of altered migration patterns of cultured T cells is clearly important if the full potential of such cells is ever to be realized in future clinical applications. Even so, despite this problem, secondary T cell populations generated *in vitro* were as potent as T cells taken from the peak of the primary response *in vivo*. This suggests the possibility of employing such cells therapeutically against certain types of viral infection in patients who are immunosuppressed. The donors of "memory" T cells to be used as "responders" *in vitro* could be HLA-compatible volunteers with an appropriate history of exposure to the specific viral infection, but cryo-stored lymphocytes taken from graft recipients prior to surgery and immunosuppressive therapy are another possibility. Finally, it would be interesting to examine the feasibility of a similar approach to immunotherapy against tumours.

#### SUMMARY

The antiviral activity of secondary effector cells generated *in vitro* against ectromelia virus infection was investigated. Depending upon the order of administration of cells and virus,  $2 \times 10^6$  cells significantly reduce virus titres in recipient mice. Mice injected with lymphocytic choriomeningitis (LCM) virus are not protected by secondary effectors against ectromelia virus infection and *vice versa*.



The cells conferring antiviral activity are sensitive to anti-theta and complement treatment and must share *H-2K* or *H-2D* region genes with the recipients in order for significant reduction of virus titres to occur.

The possibility of exploiting this approach in clinical medicine by using T cell-mediated mechanisms against certain viral infections is

briefly discussed.

- Blanden, R. V. and Langman, R. S. 1977. Cell-mediated immunity to bacterial infection in the mouse. Thymus-derived cells as effectors of acquired resistance to *Listeria monocytogenes*. *Scand. J. Immunol.* 1:373.
- Blanden, R. V., Povern, N. A., Pang, T. B., Gardner, I. D. and Parish, C. R. 1975a. Effects of thymus-independent (a) cells and the *H-2* gene complex on antiviral function of immune thymus-derived (T) cells. *Aust. J. Exp. Biol. Med. Sci.* 103:157.
- Blanden, R. V., Doherty, P. C., Dunlop, M. B. C., Gardner, I. D., Zinkernagel, R. M. and David, C. S. 1975b. Genes required for cytotoxicity against virus infected target cells in *K* and *D* regions of *H-2* complex. *Nature (London)* 254:280.
- Blanden, R. V. and Gardner, I. D. 1976. The cell-mediated immune response to ectromelia virus infection. I. Kinetics and characteristics of the primary effector T cell response *in vivo*. *Cell Immunol.* 28:271.
- Blanden, R. V., Zee, U. and Dunlop, M. B. C. 1977. *In vitro* primary induction of cytotoxic T cells against virus-infected syngeneic cells. *J. Immunol. Methods* 10:73.
- Dunlop, M. B. C. and Blanden, R. V. 1976. Secondary cytotoxic cell response to lymphocytic choriomeningitis virus. I. Kinetics of induction *in vitro* and yields of effector cells. *Immunology* 31:171.
- Dunlop, M. B. C. 1978. Secondary cytotoxic cell response to lymphocytic choriomeningitis virus. III. *In vivo* protective activity of effector cells generated *in vitro*. *Immunology* 34:291.
- Davidson, W. F. and Parish, C. R. 1975. A procedure for removing red cells and dead cells from lymphoid cell suspensions. *J. Immunol. Methods* 7:291.
- Gardner, I., Povern, N. A. and Blanden, R. V. 1974. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. II. Identification of effector cells and analysis of mechanisms. *Eur. J. Immunol.* 4:68.

## REFERENCES

- Blanden, R. V. 1970. Mechanisms of recovery from a generalized viral infection: Mousepox. I. The effects of antithymocyte serum. *J.Exp.Med.* 132:1035.
- Blanden, R. V. 1971a. Mechanisms of recovery from a generalized viral infection: Mousepox. II. Passive transfer of recovery mechanisms with immune lymphoid cells. *J.Exp.Med.* 133:1074.
- Blanden, R. V. 1971b. Mechanisms of recovery from a generalized viral infection: Mousepox. III. Regression of infectious foci. *J.Exp.Med.* 133:1090.
- Blanden, R. V. and Langman, R. E. 1972. Cell-mediated immunity to bacterial infection in the mouse. Thymus-derived cells as effectors of acquired resistance to *Listeria monocytogenes*. *Scand.J.Immunol.* 1:379.
- Blanden, R. V., Bower, N. A., Pang, T. E., Gardner, I. D. and Parish, C. R. 1975a. Effects of thymus-independent (B) cells and the H-2 gene complex on antiviral function of immune thymus-derived (T) cells. *Aust.J.Exp.Biol.Med.Sci.* 53:187.
- Blanden, R. V., Doherty, P. C., Dunlop, M. B. C., Gardner, I. D., Zinkernagel, R. M. and David, C. S. 1975b. Genes required for cytotoxicity against virus infected target cells in K and D regions of H-2 complex. *Nature(London)* 254:269.
- Blanden, R. V. and Gardner, I. D. 1976. The cell-mediated immune response to ectromelia virus infection. I. Kinetics and characteristics of the primary effector T cell response *in vivo*. *Cell.Immunol.* 22:271.
- Blanden, R. V., Kees, U. and Dunlop, M. B. C. 1977. *In vitro* primary induction of cytotoxic T cells against virus-infected syngeneic cells. *J.Immunol.Methods* 16:73.
- Dunlop, M. B. C. and Blanden, R. V. 1976. Secondary cytotoxic cell response to lymphocytic choriomeningitis virus. I. Kinetics of induction *in vitro* and yields of effector cells. *Immunology* 31:171.
- Dunlop, M. B. C. 1978. Secondary cytotoxic cell response to lymphocytic choriomeningitis virus. III. *In vivo* protective activity of effector cells generated *in vitro*. *Immunology* 34:291.
- Davidson, W. F. and Parish, C. R. 1975. A procedure for removing red cells and dead cells from lymphoid cell suspensions. *J.Immunol.Methods* 7:291.
- Gardner, I., Bower, N. A. and Blanden, R. V. 1974. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. II. Identification of effector cells and analysis of mechanisms. *Eur.J.Immunol.* 4:68.



- Gardner, I. D. and Blanden, R. V. 1976. The cell-mediated immune response to actromelia virus infection. II. Secondary response *in vitro* and kinetics of memory T cell production *in vivo*. *Cell.Immunol.* 22:283.
- Kees, U. and Blanden, R. V. 1976. A single genetic element in *H-2K* affects mouse T-cell antiviral function in poxvirus infection. *J.Exp.Med.* 143:450.
- Pang, T. and Blanden, R. V. 1977. Genetic factors in the stimulation of T cell responses against ectromelia virus-infected cells: Role of *H-2K*, *H-2D* and *H-2I* genes. *Aust.J.Exp.Biol.Med.Sci.* 55:549.
- Zinkernagel, R. M., Pang, T. and Blanden, R. V. 1975. Effect of cell dose and dose of infectious agent on expression of protection against *Listeria monocytogenes* and ectromelia virus in cell transfer models. *Infect.Immun.* 11:1170.



FIG. 1 - Relationship between dose of secondary effector cells injected and protection from ectromelia infection, expressed as reduction below control level in mean  $\log_{10}$  virus titer in groups of four recipient spleens per point.



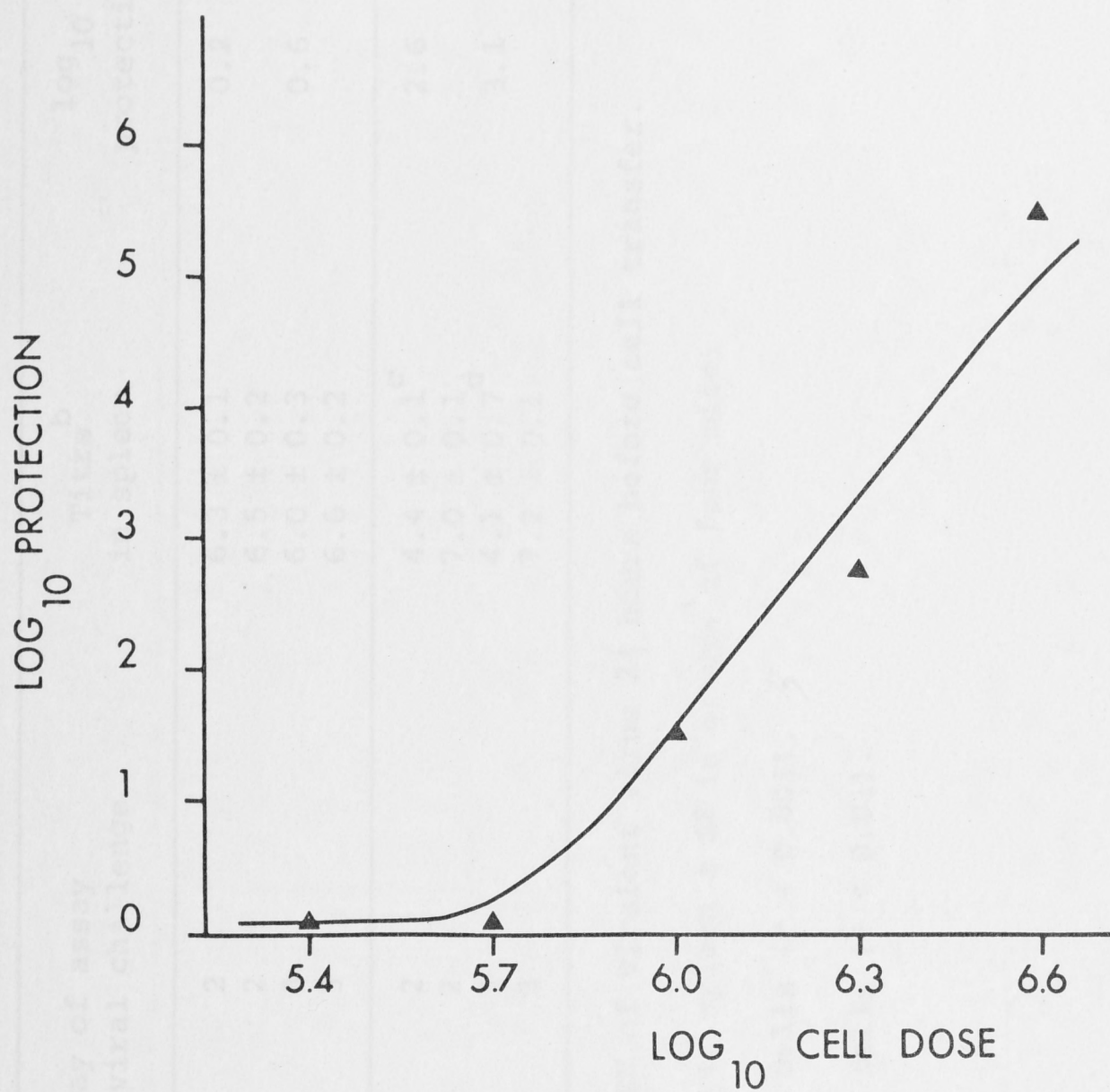


FIG. 1 - Relationship between dose of secondary effector cells injected and protection from ectromelia infection, expressed as reduction below control level in mean  $\log_{10}$  virus titre in groups of four recipient spleens per point.

TABLE 1

ANTIVIRAL EFFECT OF SECONDARY ECTROMELIA IMMUNE CELLS<sup>a</sup>

Experiment	Cell dose transferred	Day of assay post viral challenge	Titre <sup>b</sup> in spleen	log <sub>10</sub> protection
1	1.5 x 10 <sup>7</sup>	2	6.3 ± 0.1	0.2
	nil	2	6.5 ± 0.2	
	1.5 x 10 <sup>7</sup>	3	6.0 ± 0.3	0.6
	nil	3	6.6 ± 0.2	
2	8 x 10 <sup>7</sup>	2	4.4 ± 0.1 <sup>c</sup>	2.6
	nil	2	7.0 ± 0.1	
	8 x 10 <sup>7</sup>	3	4.1 ± 0.7 <sup>d</sup>	3.1
	nil	3	7.2 ± 0.1	

a Recipients were injected i.v. with 2.5 x 10<sup>6</sup> PFU of virulent virus 24 hours before cell transfer.

b Results are expressed as mean log<sub>10</sub> virus PFU per spleen ± SE in groups of four mice.

c Significantly less than control group given no cells (P < 0.001).

d Significantly less than control group given no cells (P < 0.01).



TABLE 2

PROTECTIVE EFFECT OF A LOW SECONDARY CELL DOSE IS DEPENDENT UPON ORDER OF ADMINISTRATION<sup>a</sup>

Protocol day 0	day 1	day 2	day 3	Spleen titres <sup>b</sup>	log <sub>10</sub> protection
virus	cells	*		5.8 ± 0.1	0.4
virus ) cells )		*		4.9 ± 0.2 <sup>c</sup>	1.3
cells	virus		*	4.0 ± 0.2 <sup>d</sup>	2.2
virus		*		6.2 ± 0.1	

a Recipients were injected i.v. with  $5 \times 10^5$  PFU of virulent virus and  $2 \times 10^6$  effector cells.

\* Day of assay of organ.

b Results are expressed as mean log<sub>10</sub> virus PFU per spleen ± SE in groups of four mice.

c Significantly less than control group given no cells ( $P < 0.01$ ).

d Significantly less than control group given no cells ( $P < 0.001$ ).



TABLE 3A

ANTIVIRAL ACTIVITY *IN VIVO* OF  
C57Bl/6 EFFECTOR CELLS

Cell transfer *in vivo*:<sup>a</sup>

Cells transferred	Spleen titres <sup>b</sup> in recipients
4 x 10 <sup>6</sup> secondary effectors	2.6 ± 0.2 <sup>c</sup>
4 x 10 <sup>6</sup> primary effectors	3.0 ± 0.3 <sup>c</sup>
4 x 10 <sup>6</sup> normal spleen cells	6.1 ± 0.2

a Recipients were injected i.v. with 2.5 x 10<sup>6</sup> PFU virulent ectromelia virus 24 hours after transfer of cells and splenic titres were determined 48 hours after virus injection.

b Results are expressed as mean log<sub>10</sub> virus PFU per spleen ± SE in groups of four mice.

c Significantly less than control group given normal cells (P < 0.001).

TABLE 3B

CYTOTOXIC ACTIVITY *IN VITRO* OF  
C57Bl/6 EFFECTOR CELLS

Cytotoxicity assay *in vitro*:

Cells	Killer: target ratio	% <sup>51</sup> Cr release from C57Bl/6 macrophage targets <sup>d</sup>	
		ectromelia infected	uninfected
secondary effectors	1:1	43.5 <sup>e</sup>	4.5
	4:1	66.0 <sup>e</sup>	2.2
primary effectors	1:1	13.8 <sup>f</sup>	4.8
	4:1	39.1 <sup>e</sup>	5.5
normal spleen cells	1:1	0.0	0.0
	4:1	0.0	0.5

d Data given are means of triplicates with spontaneous release subtracted.

e Significantly more lysis than uninfected target cells at the same killer:target ratio (P < 0.001).

f Significantly more lysis than uninfected target cells at the same killer:target ratio (P < 0.01).

TABLE 4

ANTIVIRAL EFFECT IS ABROGATED AFTER TREATMENT OF SECONDARY  
EFFECTOR CELLS WITH ANTI-THETA ASCITIC FLUID AND COMPLEMENT<sup>a</sup>

Cells transferred	Treatment	Spleen titres <sup>b</sup>
$2 \times 10^6$	nil	$3.1 \pm 0.2^c$
$2 \times 10^6$	normal ARR ascitic fluid and compl.	$2.9 \pm 0.2^c$
$2 \times 10^6$	anti-theta ascitic fluid and compl.	$4.5 \pm 0.2$
nil	-	$4.7 \pm 0.1$

a Recipients were injected i.v. with  $6.6 \times 10^5$  PFU virulent virus 24 hours after injection of cells and splenic titres were determined 48 hours after virus injection.

b Results are expressed as mean  $\log_{10}$  virus PFU per spleen  $\pm$  SE in groups of four mice.

c Significantly less than control group given no cells ( $P < 0.001$ ).



TABLE 5

ANTIVIRAL EFFECT OF SECONDARY EFFECTOR T CELLS IS H-2 RESTRICTED<sup>a</sup>

Effector cell donors	H-2-map <sup>b</sup>	Recipients		Spleen titres <sup>c</sup> in recipients
		Strain	H-2-map	
A.TL	skkkkd	SJL	ssssss	4.6 ± 0.6 <sup>d</sup>
A.TL	skkkkd	CBA	kkkkkk	6.4 ± 0.1
A.TL	skkkkd	BALB/c	dddddd	5.0 ± 0.3 <sup>e</sup>
CBA	kkkkkk	CBA	kkkkkk	4.0 ± 0.5 <sup>d</sup>
nil		SJL	ssssss	6.2 ± 0.2
nil		CBA	kkkkkk	5.8 ± 0.4
nil		BALB/c	dddddd	6.8 ± 0.2

a Recipients were given  $3 \times 10^6$  effector cells i.v. and 24 hours later  $5 \times 10^5$  PFU of virulent virus. Virus titres in spleens were determined 48 hours after injection.

b H-2 maps are for *K*, *I-A*, *I-B*, *I-C*, *S* and *D* region of the gene complex.

c Results are expressed as mean  $\log_{10}$  virus PFU per spleen ± SE in groups of four mice.

d Significantly less than syngeneic control group given no cells ( $P < 0.001$ ).

e Significantly less than syngeneic control group given no cells ( $P < 0.01$ ).



TABLE 6

VIRUS SPECIFICITY OF SECONDARY EFFECTOR CELLS IN CYTOTOXICITY ASSAY<sup>a</sup>

Cells	Killer: target ratio	L-929 targets		
		ectromelia-inf.	LCM-inf.	uninf.
Secondary anti-ectromelia effectors	2:1	22.5 <sup>b</sup>	2.1	1.6
Secondary anti-LCM effectors	1:1	0.0	23.4 <sup>b</sup>	0.2

- a Mean % <sup>51</sup>Cr release from groups of three wells, spontaneous lysis subtracted.
- b Significantly higher lysis than uninfected controls and control infected with the other virus, or infected targets attacked by T cells from cultures containing the other virus (P < 0.001).

n.d. = not determined

a Cell dose transferred was  $2 \times 10^6$ .

b Recipients were infected i.v. with  $2.5 \times 10^6$  PFU virulent ectromelia virus or with 2500 PFU W83 LCM virus, 24 hours after transfer of cells and splenic virus titres were determined 48 hours after injection.

c Results are expressed as mean  $\log_{10}$  virus PFU per spleen  $\pm$  SD in groups of four mice.

d Significantly less than control group given no cells and injected with the same virus (P < 0.01).

TABLE 7

UNIDIRECTIONAL VIRUS SPECIFICITY OF PROTECTIVE EFFECT OF SECONDARY T  
CELLS USING ECTROMELIA AND WE3 LCM VIRUS FOR T CELL STIMULATION

Specificity of T cells transferred <sup>a</sup>	Virus injected <sup>b</sup> into recipients	Spleen titres <sup>c</sup>	
		ectromelia	LCM
nil	ectromelia	6.5 ± 0.2	n.d.
anti-ectromelia effectors	ectromelia	1.5 ± 0.1 <sup>d</sup>	n.d.
anti-LCM effectors	ectromelia	4.3 ± 0.3 <sup>d</sup>	n.d.
nil	LCM	n.d.	6.2 ± 0.5
anti-ectromelia effectors	LCM	n.d.	6.1 ± 0.1
anti-LCM effectors	LCM	n.d.	4.5 ± 0.9 <sup>d</sup>

n.d. = not determined

a Cell dose transferred was  $2 \times 10^6$ .

b Recipients were injected i.v. with  $2.5 \times 10^6$  PFU virulent ectromelia virus or with 2500 PFU WE3 LCM virus, 24 hours after transfer of cells and splenic virus titres were determined 48 hours after injection.

c Results are expressed as mean  $\log_{10}$  virus PFU per spleen ± SE in groups of four mice.

d Significantly less than control group given no cells and injected with the same virus ( $P < 0.01$ ).



TABLE 8

BIDIRECTIONAL VIRUS SPECIFICITY OF PROTECTIVE EFFECT OF SECONDARY T CELLS USING ECTROMELIA AND ARMSTRONG LCM VIRUS FOR T CELL STIMULATION

Cells transferred <sup>a</sup>	Virus injected <sup>b</sup>	Titre <sup>c</sup>	
		ectromelia	LCM
nil	ectromelia	6.9 ± 0.1	n.d.
ectromelia secondary effectors	ectromelia	5.2 ± 0.3 <sup>d</sup>	n.d.
ectromelia primary effectors	ectromelia	5.9 ± 0.2 <sup>d</sup>	n.d.
LCM secondary effectors	ectromelia	6.7 ± 0.1	n.d.
nil	LCM	n.d.	6.3 ± 0.1
ectromelia secondary effectors	LCM	n.d.	6.0 ± 0.1
LCM primary effectors	LCM	n.d.	3.8 ± 0.3 <sup>d</sup>

n.d. = not determined

a Cell dose transferred was  $2 \times 10^6$ .

b Recipients were injected i.v. with  $2.5 \times 10^6$  PFU virulent ectromelia virus or with 2500 PFU WE3 LCM virus 24 hours after transfer of cells and splenic virus titres were determined 48 hours after injection.

c Results are expressed as mean  $\log_{10}$  virus PFU per spleen ± SE in groups of four mice.

d Significantly less than control groups given no cells and injected with the same virus ( $P < 0.01$ ).



## INTRODUCTION

## CHAPTER 4

Cytotoxic thymus-derived lymphocytes (T cells) are a prominent part of the immune response to several groups of viruses (Blanden *et al.*, 1977; Doherty *et al.*, 1976), and in some cases seem to be essential elements in the process of recovery from primary infection (Blanden, 1974; Kees and Blanden, 1976). An intriguing aspect of these T cells is that they apparently recognize both virus-specific and host-specific components in the antigenic patterns on infected cells (Doherty and Zinkernagel, 1974; Zinkernagel and Doherty, 1974). In mice, the host-specific components seem to be coded by gene(s) in either the H-2K or H-2D region of the major histocompatibility complex (MHC) (Blanden *et al.*, 1975). Further investigation of this class of T cell response is therefore indicated, with the dual objectives of assessing their potential therapeutic importance (in viral infection) on the one hand, and further elucidation of the role of the MHC in cellular interactions on the other hand. Since *in vitro* systems offer far more control over experimental variables concerned with cellular and molecular mechanisms in the immune response than *in vivo* systems, we have been exploring *in vitro* methods for the generation of cytotoxic T cells against virus-infected syngeneic cells. Although secondary responses had been readily achieved (Garner and Blanden, 1976; Denlip and Blanden, 1976), primary responses were not previously detected by such methods. We report here on the variables influencing primary cytotoxic T cell responses *in vitro* using cells infected with either reovirus or lymphocytic choriomeningitis (LCM) virus as the stimulus.

IN VITRO PRIMARY INDUCTION OF CYTOTOXIC  
T CELLS AGAINST VIRUS-INFECTED SYNGENEIC  
CELLS

## MATERIALS AND METHODS

## Mice

Inbred mice were bred at the John Curtin School and used at 6-8 weeks of age. In any one experiment, mice of the same sex were used.

## INTRODUCTION

Cytotoxic thymus-derived lymphocytes (T cells) are a prominent part of the immune response to several groups of viruses (Blanden *et al.*, 1977; Doherty *et al.*, 1976), and in some cases seem to be essential elements in the process of recovery from primary infection (Blanden, 1974; Kees and Blanden, 1976). An intriguing aspect of these T cells is that they apparently recognize both virus-specific and host-specific components in the antigenic patterns on infected cells (Doherty and Zinkernagel, 1974; Zinkernagel and Doherty, 1974). In mice, the host-specific components seem to be coded by gene(s) in either the *H-2K* or *H-2D* region of the major histocompatibility complex (MHC) (Blanden *et al.*, 1975). Further investigation of this class of T cell response is therefore indicated, with the dual objectives of assessing their potential therapeutic importance (in viral infection) on the one hand, and further elucidation of the role of the MHC in cellular interactions on the other hand. Since *in vitro* systems offer far more control over experimental variables concerned with cellular and molecular mechanisms in the immune response than *in vivo* systems, we have been exploring *in vitro* methods for the generation of cytotoxic T cells against virus-infected syngeneic cells. Although secondary responses had been readily achieved (Gardner and Blanden, 1976; Dunlop and Blanden, 1976), primary responses were not previously detected by such methods. We report here on the variables influencing primary cytotoxic T cell responses *in vitro* using cells infected with either ectromelia or lymphocytic choriomeningitis (LCM) virus as the stimulus.

## MATERIALS AND METHODS

### Mice

Inbred mice were bred at the John Curtin School and used at 6 - 8 weeks of age. In any one experiment, mice of the same sex were used.



CBA/H, BALB/c and C57BL/6 strains were bred and reared under specific pathogen-free conditions, whilst B6.C-H-2<sup>ba</sup>, B10.A, B10.A(2R) and B10.A(5R) were bred and reared under conventional conditions.

### Viruses

Stocks of attenuated (Hampstead egg strain) and virulent (Moscow strain) ectromelia virus were prepared and titrated as described in Chapter 2, and amounts were expressed as plaque-forming units (PFU). The stocks of WE3 strain LCM virus used were from guinea pig lung or spleen and are described in Chapter 3.

### Tissue culture medium

The complete medium routinely used was GIBCO Eagle's minimal essential medium with non-essential amino acids (F-15) with 10% foetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin,  $10^{-4}$  M 2-mercaptoethanol (2ME), and buffered with sodium bicarbonate to give pH = 7.0 in a gas mixture of 10% CO<sub>2</sub> and 7% O<sub>2</sub> in N<sub>2</sub>. A selected batch of FCS (Commonwealth Serum Laboratories, Melbourne, Australia) was used in primary response cultures unless otherwise stated. FCS and 2ME were added to the medium immediately before use. The basic medium (F-15) could be stored at 4°C for up to 2 weeks. In cytotoxicity assays, 2ME was omitted from medium used and unselected FCS was also used since different batches had no influence on results.

### Primary response cultures

Spleen cells, lymph node cells (from popliteal, inguinal and aortic nodes) and peritoneal cells were obtained as described (Blanden and Langman, 1972; Blanden and Mims, 1973). Briefly, spleens and lymph nodes were cut into pieces and pressed through stainless steel sieves into Puck's A saline (0.8% NaCl, 0.04% KCl, 0.1% glucose, 0.035% NaHCO<sub>3</sub>, 0.002% phenol red in deionized water). Cell clumps were dissociated by

pipetting and capsular and fibrous material was removed by suspending the crude preparation in Puck's A saline at pH 8.0 and centrifuging gently for 30 seconds, which left the cells in suspension. Peritoneal cells were obtained by injecting 2-4 ml of ice-cold Puck's A saline intraperitoneally into mice and then aspirating the cell-rich suspension. All cell preparations were washed 2-3 times and finally suspended in complete F-15 with 10% FCS.

Spleen or peritoneal cells to be used as "stimulators" were infected with virus as follows: Moscow strain ectromelia was added to spleen cell suspensions (ultimately at  $10^8$  cells/ml) at 5 PFU/cell. After 60 minutes at  $37^{\circ}\text{C}$  the cells were washed 3 times in complete F-15 at  $37^{\circ}\text{C}$ , left for 3 hours at  $37^{\circ}\text{C}$  at pH 7.0 in the appropriate gas mixture, then washed 3 more times before addition to "responder" cells. For peritoneal cells, infection was for 30 minutes at 5 PFU/cell and  $5 \times 10^7$  cells/ml. After washing 3 times in complete F-15 at  $4^{\circ}\text{C}$ , cells were dispensed at the appropriate density into tissue culture flasks (Falcon Plastics,  $75\text{ cm}^2$  or  $25\text{ cm}^2$ ), left for 3 hours at  $37^{\circ}\text{C}$  and pH 7.0 to allow infected macrophages to adhere to the plastic, and then washed 3 times in  $37^{\circ}\text{C}$  medium to remove all non-adherent cells before addition of "responder" cells. Similar procedures were followed with LCM virus, except that undiluted virus stocks were used to infect, and 18 hours elapsed between infection and addition to responders. After responder cells were added (final concentration usually  $2 \times 10^6$ /ml in 10 ml/ $25\text{ cm}^2$  flask and 40 ml/ $75\text{ cm}^2$  flask) cultures were incubated at  $39^{\circ}\text{C}$  in the case of ectromelia virus and  $37^{\circ}\text{C}$  with LCM virus at pH 7.0 in complete medium for several days before harvesting of responders and assaying for cytotoxic activity.  $39^{\circ}\text{C}$  is non-permissive for ectromelia replication and thus prevents the cytopathic effects of



this virus against responder cells, which are expressed at 37°C (Gardner and Blanden, 1976). LCM virus is not cytopathic for murine lymphoid cells.

#### Removal of macrophages from responder cells

Responder cells at  $2 \times 10^7$ /ml in 5-7 ml of complete F-15 containing 100-120 mg of carbonyl iron were agitated on a rotator for 60 minutes at 37°C. Cells associated with the iron (mainly macrophages) were then deposited by a magnet and the supernatant containing non-adherent cells free of macrophages was then used.

#### Removal of dead cells

After harvesting of responder cells from primary responses, dead cells were removed by centrifuging through Isopaque/Ficoll as described in Chapter 3. The cells were then washed twice, live cells counted, and added to labelled target cells for cytotoxicity assays. Yields of responder cells given in some tables are percentage of live cells recovered after culture relative to the number of live responder cells originally cultured. Yields were generally higher with LCM than with ectromelia. Whether this is related to the 39°C incubation or toxic effects of ectromelia virus was not investigated further.

#### Anti-theta treatment

Use of anti-theta and rabbit complement treatment is described in Chapter 3.

#### Cytotoxicity assays

A  $^{51}\text{Cr}$  release assay employing peritoneal macrophage or L929 target cells was employed. These methods are also expounded in Chapter 3. Results were expressed as mean percentage of targets lysed in triplicate assay wells. Standard errors of the mean were usually less than 3% and are omitted from tables for clarity. Significance was determined

by Student's t test.

## RESULTS

### General

As the total number of variables in any *in vitro* immune response system is very large, we do not intend to describe experiments dealing with all of them.

Fortuitously, it was found that C57BL/6 mice gave superior responses to CBA/H and BALB/c mice. Under certain conditions, C57BL/6 gave good responses whilst CBA/H responses were undetectable. Data illustrating this point will be described below. For this reason, mainly C57BL/6 mice were used in characterizing the conditions needed and the basic parameters of the response.

On the basis of previous experience with secondary responses *in vitro* (Gardner and Blanden, 1976; Dunlop and Blanden, 1976), the precedents set by others studying primary T cell responses *in vitro* to non-infectious antigens (Shearer, 1974; Plata *et al.*, 1975; Burton *et al.*, 1975) and our own preliminary experiments with primary responses, we routinely used F-15 medium with 10% FCS, antibiotics,  $10^{-4}$  M 2ME and responder cell densities of  $2 \times 10^6$ /ml as described in Materials and Methods.

Preliminary experiments with uninfected "stimulators" gave low cytotoxic activity against both uninfected and infected target cells. This apparent "autoreactivity" of cultured lymphoid cells was always seen if high effector:target ratios were used; it has been described by others (Melief *et al.*, 1975) and will not be pursued further here. It may well reflect activation of T cells against antigens from the medium (Forni and Green, 1976).

Removal of dead cells by the Isopaque/Ficoll method (Davidson and



Parish, 1976) from responder cells harvested after primary responses to infected stimulators gave up to 50% improvement in expression of cytotoxicity against infected target cells and therefore was always employed. Conditions for the cytotoxicity assay using macrophage target cells

Since in most experiments C57BL/6 cells were used in primary *in vitro* responses, macrophage target cells were employed in the  $^{51}\text{Cr}$  assay. The kinetics of the cytotoxic effect of ectromelia-immune effector cells from primary cultures of C57BL/6 cells (as described in Materials and Methods) were determined at an effector:target ratio of 12:1. Figure 1 illustrates that the percentage of  $^{51}\text{Cr}$  released by effector cells (specific release) from both infected and uninfected target cells increased linearly with time. A plateau was reached 8-10 hours after the interaction began. The release from uninfected and infected target cells was markedly increased after an incubation time of 18 hours, so subsequent assays of primary effector cells from cultures were run for 8 hours, since the difference between infected and uninfected target cells was maximal at this time.

To further optimize the chromium release assay, the multiplicity of infection (MOI) used for macrophage target cells was graded from 20 PFU/cell to 100 PFU/cell. Representative results using spleen cells from CBA/H mice given  $2 \times 10^4$  PFU of attenuated ectromelia virus intravenously (Blanden and Gardner, 1976) are listed in Table 1. They clearly show that the specific release is dependent on the MOI used for the target cells. In similar experiments using macrophages from other strains of mice, we observed a marked increase in the spontaneous release (incubation of target cells with medium only) at the highest MOI employed, therefore in all subsequent experiments macrophage target cells were infected with 50 PFU/cell.

#### FCS concentration and volume of medium

Primary responses were initiated *in vitro* in 25 cm<sup>2</sup> flasks using C57BL/6 mice as cell donors. The medium contained either 5% or 10% FCS and the final volume of medium was either 10 ml or 20 ml per flask with responder cells (a mixture of 7 parts spleen to 1 part lymph node) at  $2 \times 10^7$ /flask. Responder:stimulator ratio was 4:1. Cells were harvested after 5 days and assayed for cytotoxicity against infected and uninfected C57BL/6 macrophage target cells. The results showed that varying the FCS concentration and volume of medium had little effect on the generation of cytotoxic activity against infected target cells, but the yields of responder cells were highest with 10% FCS and 10 ml/flask (data not shown). Consequently in subsequent experiments, 10% FCS and volumes of 10 ml/25 cm<sup>2</sup> flask and 40 ml/75 cm<sup>2</sup> flask were used. Further post-harvest culturing of responders at high density in fresh medium for 24 hours as described by Burton *et al.* (1975) caused no improvement in cytotoxic activity (data not shown).

#### Batch of FCS

Four different batches of FCS were screened for their effects in primary responses in 75 cm<sup>2</sup> flasks using C57BL/6 responder cells and ectromelia-infected syngeneic spleen cells as stimulators. Responder cells were a mixture of 7 parts spleen to 1 part lymph node and responder:stimulator ratio was 4:1. Responders were harvested at 5 days and assayed on syngeneic macrophage target cells. Three out of the 4 batches of FCS gave satisfactory responses as indicated by more lysis of infected than uninfected targets, but the fourth batch was unsatisfactory (Table 2). One of the satisfactory batches was used in all subsequent experiments.



### Responder cell density

The optimal responder cell density was determined by culturing together varying numbers of C57BL/6 responder and syngeneic ectromelia-infected stimulator cells at a constant responder to stimulator ratio of 4:1 (Table 3). A responder cell density of  $2 \times 10^6$  cells/ml gave the best results. The  $^{51}\text{Cr}$  release from uninfected target cells was markedly increased if higher responder cell densities were used in culture. Cultures were routinely set up at a responder cell density of  $2 \times 10^6$  cells/ml.

### Kinetics

Primary responses were initiated in  $75 \text{ cm}^2$  flasks using populations of C57BL/6 responder and ectromelia-infected stimulator cells. Responder:stimulator ratio was 4:1. Responders were harvested at different times after initiation and assayed on C57BL/6 macrophage targets. Significant lysis of infected targets occurred with cells harvested on days 5 and 6; though the response was not evident on day 4 (Table 4). Harvesting was therefore done on days 5 or 6 in further experiments, to avoid the potential problems of medium exhaustion and falling live cell yield with time. Tables 2 to 4 illustrate that different cell populations could act as responders but no clear basis for choice was evident.

### Composition of responder and stimulator cell populations

Further investigations of the effects of varying stimulator and responder cell type were done with ectromelia virus in  $25 \text{ cm}^2$  flasks (Table 5). With C57BL/6 cells, spleen cells alone or mixtures of spleen and lymph node (7:1) as responders gave similar specific lysis; higher proportions of lymph node cells in mixtures gave poorer responses (data not shown). A more important variable was stimulator cell type.

Macrophages were clearly superior to spleen cells, which in turn were superior to irradiated spleen cells when compared within individual experiments (i.e., when a particular pool of cells was used as responders or stimulators). However, different pools of cells (e.g., compare Experiments 1 and 2 in Table 5) gave responses of different potency. This suggested that different individual mice may influence the behaviour of cell pools to which they contribute. Tests of this proposition do indicate differences between individual mice from different cages, but it is not a major factor and will not be described further here.

Responder:stimulator ratios and stimulator cell type in relation to mouse strain

With cells from C57BL/6 mice responder:stimulator ratios of up to 15:1 using macrophage stimulators (data not shown), and 5:1 using spleen stimulators all gave satisfactory responses and a ratio of 4:1 was routinely used (Tables 2 to 5). However, cells from the two other strains of mice tested so far (CBA/H and BALB/c) seem more demanding. Table 6 shows that satisfactory responses with BALB/c cells required low responder:stimulator ratios, even with infected macrophages, the best response occurring at 2:1.

A more extensive investigation was carried out with CBA/H mice. Previous experience with secondary responses of CBA/H spleen cells *in vitro* (Pang and Blanden, 1976) had shown that the number of macrophages in the culture was a crucial factor in optimizing the response. Table 7 shows that with infected spleen cell stimulators, no significant activity against infected targets (over and above uninfected control targets) was detectable at the effector:target ratio used, despite manipulation of the macrophage content of the cultures. With macrophages as infected stimulators, however, good responses were obtained. Most activity was generated at the lowest responder:stimulator ratio used (3:2) in cultures



supplemented by  $6 \times 10^5$  uninfected macrophages per flask, each flask containing  $1.5 \times 10^7$  responders in 10 ml of medium. In this experiment, spleen cells from the peak (5 days) of the CBA/H primary response *in vivo* to  $2 \times 10^4$  PFU of attenuated ectromelia given intravenously (Blanden and Gardner, 1976) were included for comparison. At an effector:target ratio of 11:1, these cells gave 38.0% lysis of infected and 3.8% lysis of uninfected targets, virtually identical activity to the best *in vitro* response.

#### Need for macrophages and effect of target cell type

CBA/H cells were used to further confirm the dependence on macrophages of the primary response *in vitro* and to determine the sensitivity of different target cells (Table 8). Removal of macrophages from the responding cells decreased both yield and cytotoxic potency of the culture. Furthermore, infected macrophages were about 4-fold more sensitive as target cells than infected L929 cells, and uninfected macrophage controls were lysed less than uninfected L929 cells.

#### Potency of responses *in vivo* and *in vitro*

As mentioned in the case of CBA/H above, C57BL/6 primary responses *in vitro* also gave cytotoxic cell populations of similar potency to those harvested from the spleen at the peak of the primary response *in vivo* (Table 9). The secondary response *in vitro* in this experiment gave cells about 8-fold more active than those from primary responses.

#### Nature of cytotoxic cells generated in primary response *in vitro*

Cytotoxic cells generated *in vivo* during infection with ectromelia (Gardner *et al.*, 1974) and LCM virus (Doherty *et al.*, 1974) are T cells. Secondary responses *in vitro* with both viruses also generate cytotoxic T cells (Gardner and Blanden, 1976; Dunlop *et al.*, 1976). Since anti-theta and complement treatment abolished activity from primary cytotoxic cells

generated *in vitro* against both LCM (Dunlop and Blanden, 1977) and ectromelia infection (Table 10), they too appear to be T cells.

H-2 restriction of cytotoxic cells generated in primary responses *in vitro*

C57BL/6 or B6.C-H-2<sup>ba</sup> cells were used to generate primary responses to ectromelia-infected syngeneic stimulators, and cytotoxic activity was assayed on B10.A(2R), B10.A(5R) and B10.A macrophage target cells (Table 11). Significant specific lysis of infected targets over and above uninfected controls occurred only where H-2K or H-2D region genes were shared by donors of responder, stimulator and target cells. Thus C57BL/6 cultures gave cytotoxic activity against infected targets from B10.A(2R) (shared D) and B10.A(5R) (shared K, I-A, I-B) but not B10.A (shared genetic background but not H-2). B6.C-H-2<sup>ba</sup> cultures gave specific lysis only on infected B10.A(2R) targets (shared D), not B10.A(5R) (shared I-A and I-B) or B10.A. B6.C-H-2<sup>ba</sup> mice bear a mutation in K region (designated K<sup>ba</sup>) which differs from wild-type K<sup>b</sup> (Bailey *et al.*, 1971; Blanden *et al.*, 1976); the data in Table 11 thus indicate that shared K region, not I-A or I-B, was responsible for lysis of infected B10.A(5R) targets by C57BL/6 cells. This restriction of cytotoxic activity confirms that the cytotoxic cells are T cells (Zinkernagel and Doherty, 1974; Blanden *et al.*, 1975).

Virus-specificity of cytotoxic T cells generated in primary responses *in vitro*

C57BL/6 cells from responses stimulated by either ectromelia or LCM virus-infected syngeneic cells were assayed against target cells infected with either ectromelia or LCM viruses (Table 12). Only when stimulator cells and target cells were infected with the same virus did significant specific lysis occur.



## DISCUSSION

We have described here the primary generation *in vitro* of cytotoxic cells specific for virus-infected syngeneic or H-2 compatible target cells. The active cells seem to be T cells, since they are sensitive to anti-theta and complement; and they lyse only those target cells sharing H-2K or H-2D genes with the infected stimulators used to generate them, a property characteristic of murine cytotoxic T cells generated during viral infection *in vivo* (Blanden *et al.*, 1975; Doherty *et al.*, 1976). The present experiments also showed clear specificity for virus in comparisons between ectromelia and LCM viruses.

Under the optimal conditions determined for the primary response *in vitro*, the potency of anti-ectromelia cytotoxic cell populations generated over 5-6 days was virtually identical with that present at the peak of the primary response in the spleen *in vivo* after 5-6 days. The early kinetics *in vivo* and *in vitro* of the anti-ectromelia responses also seem similar, though not identical, in that activity is clearly demonstrable by day 4 *in vivo* (Blanden and Gardner, 1976), but takes a day longer to appear *in vitro*.

The conditions necessary for the *in vitro* response are basically similar to those defined for T cell responses to non-infectious antigens (Shearer, 1974; Plata *et al.*, 1975; Burton *et al.*, 1975). Eagle's minimal essential medium with non-essential amino acids, 10% FCS (of a selected batch) and  $10^{-4}$  M 2ME was used, and responder cell density was  $2 \times 10^6$ /ml, with  $2 \times 10^7$  cells occupying  $25 \text{ cm}^2$  or  $8 \times 10^7$  cells occupying  $75 \text{ cm}^2$  on the floor of plastic tissue culture flasks. The pH was maintained at about 7.0 in an atmosphere of 10%  $\text{CO}_2$  and 7%  $\text{O}_2$  in  $\text{N}_2$ . For ectromelia virus  $39^\circ\text{C}$  was essential to prevent viral replication and cytopathic effect against responding cells, but  $37^\circ\text{C}$  was employed for LCM virus. Spleen

cells alone or a mixture of 7 parts spleen to one part lymph node cells were satisfactory responders, but higher proportions of lymph node cells in the mixture were detrimental. Under more demanding conditions (irradiated stimulator cells), the mixture was superior to spleen cells as responders.

The crucial variables were the strain of mouse used, the responder:infected stimulator ratio, the type of stimulator cell, and/or the number of macrophages present in the culture. C57BL/6 cells gave satisfactory responses under a much wider variety of conditions than CBA/H or BALB/c cells. For example, they tolerated the use of infected spleen cells as stimulators, or responder:stimulator ratios as high as 15:1 using infected macrophage stimulators. CBA/H cells, on the other hand, only gave satisfactory responses with infected macrophage stimulators, and optimum responses required a responder:stimulator ratio of 2:1 or less. It was observed that the addition of uninfected macrophages to the culture improved the response. BALB/c cells were less extensively investigated, but they too gave best responses with infected macrophage stimulators and a responder:stimulator ratio of 2:1. Maximal secondary responses of CBA/H cells *in vitro* also requires that a certain number of uninfected macrophages be present (Pang and Blanden, 1976); but only 1-2% macrophages in the responder population are sufficient (Pang and Blanden, 1976) and spleen cell stimulators are satisfactory (Gardner and Blanden, 1976). Thus primary CBA/H responses demand far more rigorous control of culture conditions than secondary responses.

After harvesting of the cultures, dead cells were removed by the method of Davidson and Parish (1976). This procedure improved expression of cytotoxicity by up to 50%, possibly because some of the dead cells had been infected and thus acted as "cold" competitors in the assay against



<sup>51</sup>Cr labelled infected target cells.

The significance of the present results, in our view, is three-fold. First, the fact that potentially protective primary T cell responses against infection can now be induced *in vitro* brings us one small step closer to utilizing more fully the therapeutic power of specific cell-mediated immunity in clinical medicine. Second, the *in vitro* approach lends itself to an analysis of the cellular and genetic factors involved in the interactions between T cell subsets (Cantor and Boyse, 1975; Pang *et al.*, 1976), infected stimulator cells of different types, and accessory cells such as uninfected macrophages (Pang and Blanden, 1976). Third, it is clear that the potency of primary T cell responses against viruses, TNP (Shearer, 1974) minor histocompatibility antigens (Bevan, 1975; Gordon *et al.*, 1975) or tumor-specific antigens (Burton *et al.*, 1975) is far less than the primary response to the major alloantigens, as in mixed lymphocytic reactions (MLR). Only after priming do the responses to viruses or minor antigens approach the levels of primary MLR (Bevan, 1975; Gordon *et al.*, 1975; Gardner and Blanden, 1976; Dunlop and Blanden, 1976). If the T cell system has evolved to cope with infections or tumors, why is this so? Further investigation of the role of the MHC in T cell responses and the generation of diversity in T cell receptors (Jerne, 1971; Nabholz and Miggiano, 1977) may illuminate this intriguing question.

#### SUMMARY

An *in vitro* method is described for primary induction of murine cytotoxic T cells against syngeneic cells infected with ectromelia virus. Cytotoxicity was assayed by <sup>51</sup>Cr release from macrophage or L929 target cells. Cytotoxic activity was sensitive to anti-theta and complement and was expressed only against target cells infected with the same virus and

sharing *H-2K* or *H-2D* genes with the infected stimulator cells. The crucial factors in generating responses were mouse strain, responder:stimulator ratio, nature of infected stimulator cells, and presence of sufficient macrophages. C57BL/6 cells were less demanding than CBA/H and BALB/c cells. Under optimal conditions as defined, the *in vitro* response had similar kinetics and potency to the primary response in the spleen *in vivo*.

- Blanden, R. V. and Langman, R. W. 1973. Cell-mediated immunity to bacterial infection in the mouse. Thymus-derived cells as effectors of acquired resistance to *Listeria monocytogenes*. *Canad. J. Immunol.* 7:379.
- Blanden, R. V. and Sims, C. A. 1973. Macrophage activation in mice infected with ectromelia or lymphocytic choriomeningitis viruses. *Aust. J. Exp. Biol. Med. Sci.* 51:393.
- Blanden, R. V. 1974. T cell response to viral and bacterial infection. *Transplant. Rev.* 18:53.
- Blanden, R. V., Doherty, P. C., Dunlop, M. B. C., Gardner, I. D., Zinkernagel, R. M. and David, C. S. 1975. Genes required for cytotoxicity against virus infected target cells in K and D regions of the H-2 complex. *Nature (London)* 254:269.
- Blanden, R. V. and Gardner, I. D. 1976. The cell-mediated immune response to ectromelia virus infection. I. Kinetics and characteristics of the primary effector T cells response *in vivo*. *Cell. Immunol.* 22:277.
- Blanden, R. V., Dunlop, M. B. C., Doherty, P. C., Kohn, S. I. and McQuibban, H. F. C. 1976. Effects of four *H-2K* mutations on virus-induced antigens recognized by cytotoxic T cells. *Immunogenetics* 3:541.
- Blanden, R. V., Pang, T. B. and Dunlop, M. B. C. 1977. T cell recognition of virus-infected cells, in *Virus Infection and the cell surface*, Cell Surface Reviews, Vol. 2, G. Poste and G. L. Nicolson, eds., North Holland Publishing Company, Amsterdam, p. 249.
- Burton, R., Thompson, J. and Warner, H. L. 1975. *In vitro* induction of tumour-specific immunity. I. Development of optimal conditions for induction and assay of cytotoxic lymphocytes. *J. Immunol. Methods* 5:135.
- Cantor, H. and Boyce, E. A. 1975. Functional subclasses of different T lymphocytes bearing different Ia antigens. II. Cooperation between subclasses of  $I\gamma^+$  cells in the generation of killer activity. *J. Exp. Med.* 141:1390.
- Davidson, W. F. and Parish, C. R. 1975. A procedure for removing red cells and dead cells from lymphoid cell suspensions. *J. Immunol. Methods* 7:321.



## REFERENCES

- Bailey, D. W., Snell, G. D. and Cherry, M. 1971. Complementation and serological analysis of an *H-2* mutant, in *Immunogenetics of the H-2 system*, A. Lengerova and M. Vojtiskova, eds., Karger, Basel, p. 155.
- Bevan, M. J. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J.Exp.Med.* 142:1349.
- Blanden, R. V. and Langman, R. E. 1972. Cell-mediated immunity to bacterial infection in the mouse. Thymus-derived cells as effectors of acquired resistance to *Listeria monocytogenes*. *Scand.J.Immunol.* 1:379.
- Blanden, R. V. and Mims, C. A. 1973. Macrophage activation in mice infected with ectromelia or lymphocytic choriomeningitis viruses. *Aust.J.Exp.Biol.Med.Sci.* 51:393.
- Blanden, R. V. 1974. T cell response to viral and bacterial infection. *Transplant.Rev.* 19:56.
- Blanden, R. V., Doherty, P. C., Dunlop, M. B. C., Gardner, I. D., Zinkernagel, R. M. and David, C. S. 1975. Genes required for cytotoxicity against virus infected target cells in *K* and *D* regions of the *H-2* complex. *Nature(London)* 254:269.
- Blanden, R. V. and Gardner, I. D. 1976. The cell-mediated immune response to ectromelia virus infection. I. Kinetics and characteristics of the primary effector T cells response *in vivo*. *Cell.Immunol.* 22:271.
- Blanden, R. V., Dunlop, M. B. C., Doherty, P. C., Kohn, H. I. and McKenzie, I. F. C. 1976. Effects of four *H-2K* mutations on virus-induced antigens recognized by cytotoxic T cells. *Immunogenetics* 3:541.
- Blanden, R. V., Pang, T. E. and Dunlop, M. B. C. 1977. T cell recognition of virus-infected cells, in *Virus infection and the cell surface*, *Cell Surface Reviews*, Vol. 2, G. Poste and G. L. Nicolson, eds., North Holland Publishing Company, Amsterdam, p. 249.
- Burton, R., Thompson, J. and Warner, N. L. 1975. *In vitro* induction of tumour-specific immunity. I. Development of optimal conditions for induction and assay of cytotoxic lymphocytes. *J.Immunol.Methods* 8:133.
- Cantor, H. and Boyse, E. A. 1975. Functional subclasses of different T lymphocytes bearing different *Ly* antigens. II. Cooperation between subclasses of *Ly*<sup>+</sup> cells in the generation of killer activity. *J.Exp.Med.* 141:1390.
- Davidson, W. F. and Parish, C. R. 1975. A procedure for removing red cells and dead cells from lymphoid cell suspensions. *J.Immunol.Methods* 7:291.

- Doherty, P. C. and Zinkernagel, R. M. 1974. T cell-mediated immunopathology in viral infections. *Transplant.Rev.* 19:89.
- Doherty, P. C., Zinkernagel, R. M. and Ramshaw, I. A. 1974. Specificity and development of cytotoxic thymus-derived lymphocytes in lymphocytic choriomeningitis. *J.Immunol.* 112:1548.
- Doherty, P. C., Blanden, R. V. and Zinkernagel, R. M. 1976. Specificity of virus-immune effector T cells for H-2K or H-2D compatible interactions: Implications for H-antigen diversity. *Transplant.Rev.* 29:89.
- Dunlop, M. B. C. and Blanden, R. V. 1976. Secondary cytotoxic cell response to lymphocytic choriomeningitis virus. I. Kinetics of induction *in vitro* and yield of effector cells. *Immunology* 31:171.
- Dunlop, M. B. C., Doherty, P. C., Zinkernagel, R. M. and Blanden, R. V. 1976. Secondary cytotoxic cell response to lymphocytic choriomeningitis virus. II. Nature and specificity of effector cells. *Immunology* 31:181.
- Dunlop, M. B. C. and Blanden, R. V. 1977. Induction of a primary cytotoxic T cell response to lymphocytic choriomeningitis virus-infected cells *in vitro*. *Cell.Immunol.* 28:190.
- Forni, G. and Green, I. 1976. Heterologous sera: a target for *in vitro* cell-mediated cytotoxicity. *J.Immunol.* 116:1561.
- Gardner, I. D., Bower, N. A. and Blanden, R. V. 1974. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. *Eur.J.Immunol.* 4:63.
- Gardner, I. D. and Blanden, R. V. 1976. The cell-mediated immune response to ectromelia virus infection. II. Secondary response *in vitro* and kinetics of memory T cell production *in vivo*. *Cell.Immunol.* 22:283.
- Gordon, R. D., Simpson, E. and Samelson, L. E. 1975. *In vitro* cell-mediated immune responses to male specific (H-Y) antigen in mice. *J.Exp.Med.* 142:1108.
- Jerne, N. K. 1971. The somatic generation of immune recognition. *Eur.J.Immunol.* 1:1.
- Kees, U. and Blanden, R. V. 1976. A single genetic element in H-2K affects mouse T cell antiviral function in poxvirus infection. *J.Exp.Med.* 143:450.
- Melief, C. J. M., Schwartz, R. S., Kohn, H. I. and Melvold, R. W. 1975. Dermal histocompatibility and *in vitro* lymphocyte reactions of three new H-2 mutants. *Immunogenetics* 2:337.
- Nabholz, M. and Miggiano, V. 1977. The biological significance of the mixed leukocyte reaction, in *B and T cells in Immune Recognition*, F. Loo and G. E. Roelants, eds., E. Wiley and Sons, Chichester, England.



- Pang, T. and Blanden, R. V. 1976. The role of adherent cells in the secondary cell-mediated response *in vitro* to a natural poxvirus pathogen. *Aust.J.Exp.Biol.Med.Sci.* 54:559.
- Pang, T., McKenzie, I. F. C. and Blanden, R. V. 1976. Cooperation between mouse T cell subpopulations in the cell-mediated response to a natural poxvirus pathogen. *Cell.Immunol.* 26:153.
- Plata, F., Cerottini, J.-C. and Brunner, K. T. 1975. Primary and secondary *in vitro* generation of cytotoxic T lymphocytes in the murine sarcoma virus system. *Eur.J.Immunol.* 5:227.
- Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur.J.Immunol.* 4:527.
- Zinkernagel, R. M. and Doherty, P. C. 1974. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis virus with a syngeneic or semiallogeneic system. *Nature(London)* 248:701.

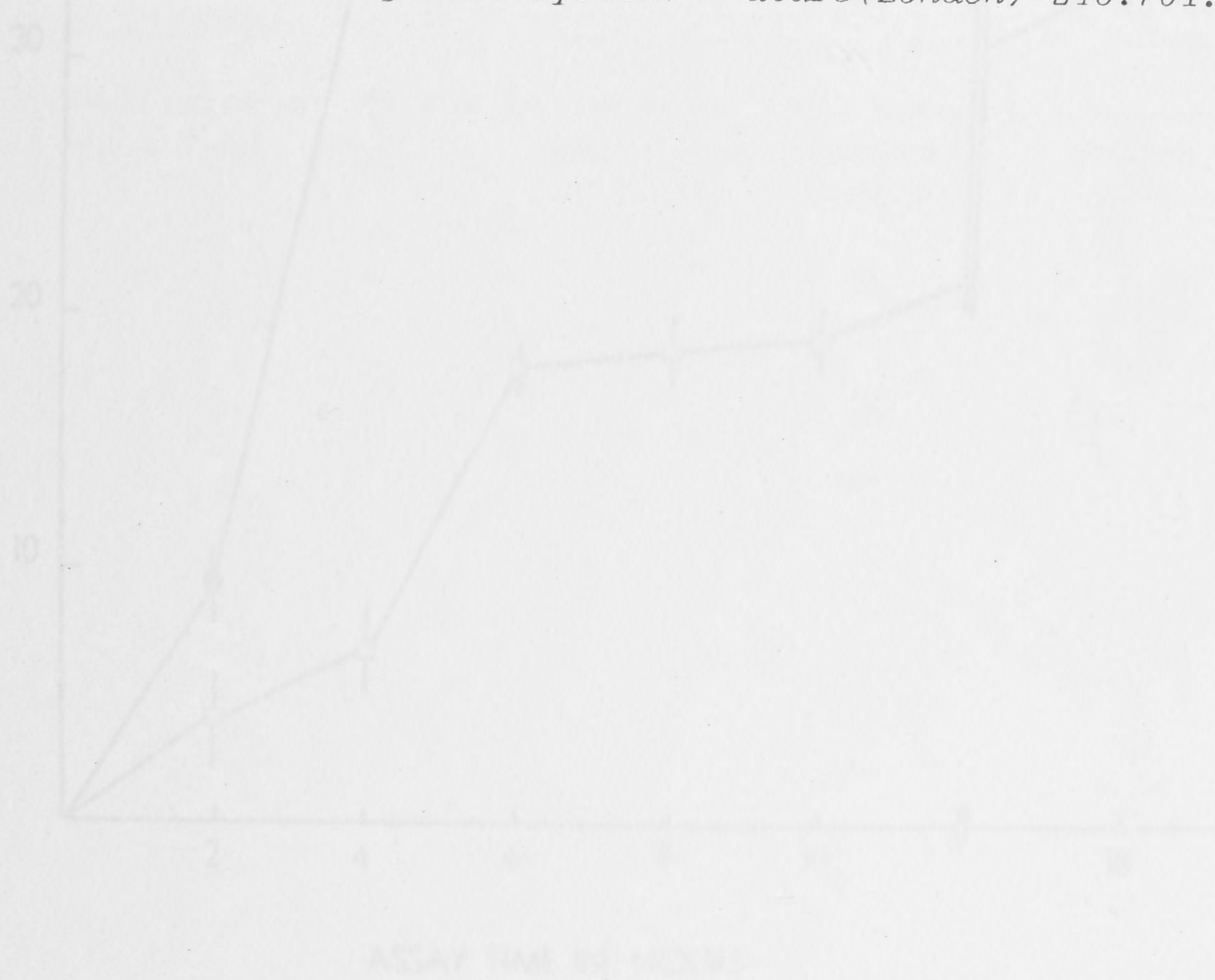


FIG. 1 - Kinetics of specific  $^{51}\text{Cr}$  release from virus-infected (□-□-□) and uninfected (O-O-O) sarcoma target cells in the presence of C57BL/6 primary effector cells from cultures at an effector:target ratio of 12:1. Each point represents the mean of 3 wells. Vertical bars indicate 1 S.D. of the mean.

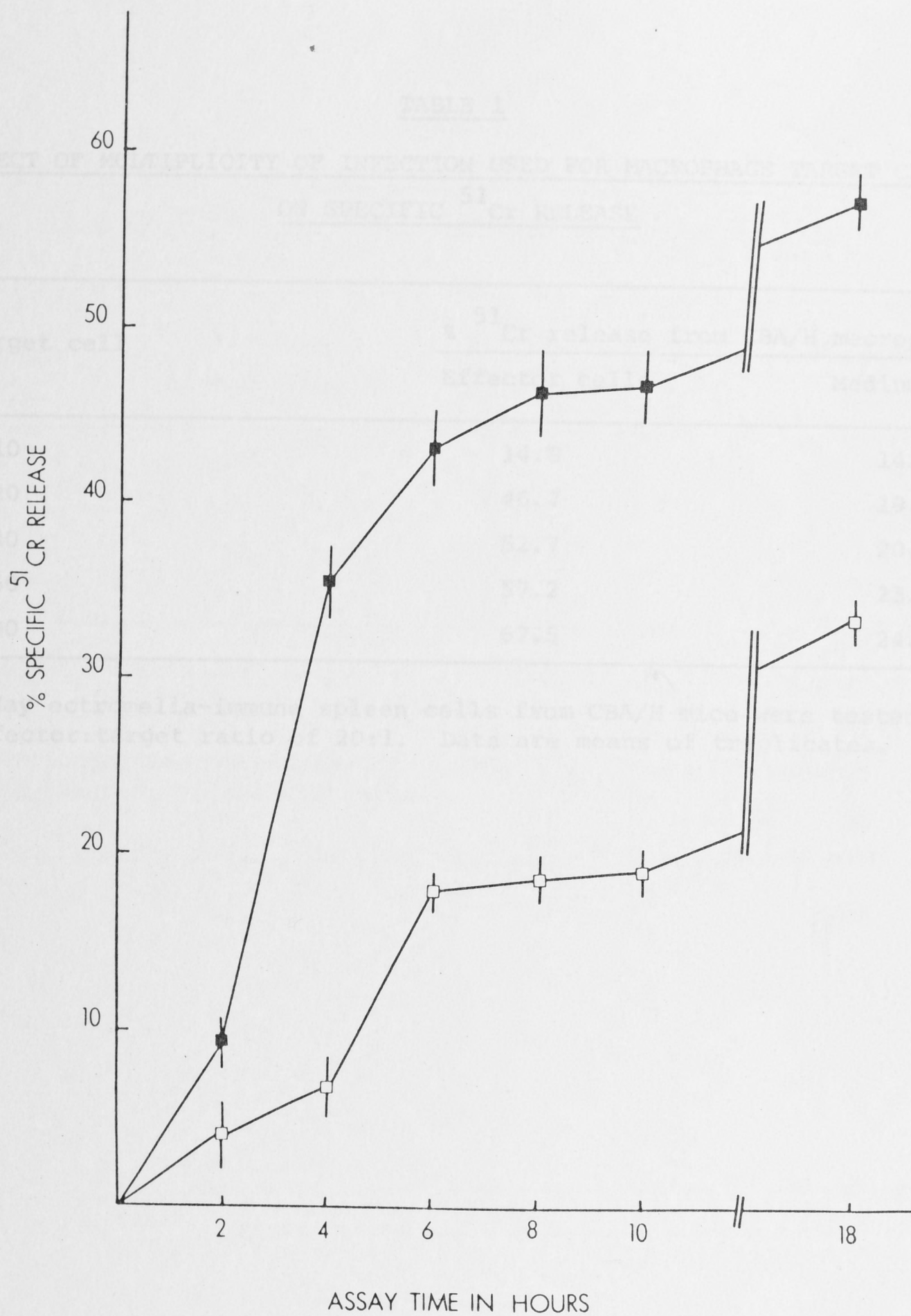


FIG. 1 - Kinetics of specific  $^{51}\text{Cr}$  release from ectromelia infected (■—■) and uninfected (□—□) C57BL/6 macrophage target cells in the presence of C57BL/6 primary effector cells from cultures at an effector:target ratio of 12:1. Each point represents the mean of 3 wells. Vertical bars enclose 2 s.e. of the mean.



TABLE 1

EFFECT OF MULTIPLICITY OF INFECTION USED FOR MACROPHAGE TARGET CELLS  
ON SPECIFIC  $^{51}\text{Cr}$  RELEASE

PFU/target cell	% $^{51}\text{Cr}$ release from CBA/H macrophages <sup>a</sup>	
	Effector cells	Medium only
10	14.8	14.3
20	46.7	19.2
40	52.7	20.6
50	57.2	23.0
100	67.5	24.4

a 5-day ectromelia-immune spleen cells from CBA/H mice were tested at an effector:target ratio of 20:1. Data are means of triplicates.

TABLE 2

EFFECT OF DIFFERENT FCS BATCHES ON PRIMARY RESPONSE *IN VITRO* OF C57BL/6  
CELLS TO SYNGENEIC ECTROMELIA-INFECTED STIMULATOR CELLS<sup>a</sup>

FCS batch	% Yield of responder cells	% <sup>51</sup> Cr release from C57BL/6 targets <sup>b</sup>	
		Infected	Uninfected
A	33.6	67.6 <sup>c</sup>	15.9
B	18.6	60.3 <sup>c</sup>	10.6
C	45.0	72.3 <sup>c</sup>	17.8
D	37.2	69.6	63.5

- a Effector cells were generated as indicated in the text and tested at an effector:target ratio of 5:1.
- b Peritoneal macrophage targets. Data are means of triplicates with spontaneous release subtracted.
- c Significantly more lysis than uninfected targets ( $P < 0.001$ ).



TABLE 3

EFFECT OF RESPONDER CELL DENSITY IN PRIMARY RESPONSE *IN VITRO* OF C57BL/6 CELLS  
TO SYNGENEIC ECTROMELIA-INFECTED STIMULATOR CELLS<sup>a</sup>

Responder cell density	% Yield of responder cells	Effector:target ratio	% <sup>51</sup> Cr release from C57BL/6 targets <sup>b</sup>	
			Infected	Uninfected
1 x 10 <sup>6</sup> /ml	5	4:1	0	7.2
		12:1	n.d. <sup>c</sup>	n.d.
2 x 10 <sup>6</sup> /ml	25	4:1	35.5	4.2
		12:1	69.8	30.2
3 x 10 <sup>6</sup> /ml	40	4:1	56.1	16.2
		12:1	81.4	53.3
4 x 10 <sup>6</sup> /ml	44	4:1	60.8	24.8
		12:1	82.4	55.1
5 x 10 <sup>6</sup> /ml	33	4:1	72.8	40.5
		12:1	89.9	71.6
6 x 10 <sup>6</sup> /ml	23	4:1	74.8	47.8
		12:1	89.7	69.3

a Responder cells were C57BL/6 spleen cells stimulated with syngeneic ectromelia-infected spleen cells at a responder:stimulator ratio of 4:1.

b Peritoneal macrophage targets. Data are means of triplicates with spontaneous release subtracted.

c n.d. = not determined.

TABLE 4

KINETICS OF PRIMARY RESPONSE *IN VITRO*<sup>a</sup> OF C57BL/6 LYMPHOID CELLS  
 AGAINST ECTROMELIA VIRUS-INFECTED SYNGENEIC CELLS

Nature of responder cells	Nature of infected stimulator cells	Day of harvest	% Yield of responder cells	Effector:target ratio	% <sup>51</sup> Cr release from targets <sup>b</sup>	
					Infected	Uninfected
Spleen + lymph node (7:1)	Spleen	4	26%	15:1	4.9	0.4
				5:1	1.0	0
		5	31%	15:1	48.4 <sup>c</sup>	15.7
				5:1	39.0 <sup>c</sup>	2.7
		6	30%	15:1	54.1 <sup>c</sup>	34.7
				5:1	44.8 <sup>c</sup>	12.3

a Cultures set up as described in Materials and Methods, responder:stimulator ratio was 4:1.

b Peritoneal macrophage targets. Data are means of triplicates with spontaneous release subtracted.

c Significantly more lysis than uninfected targets at the same effector:target ratio ( $P < 0.001$ ).



- a C57BL/6 cells were cultured for 5 days at a responder:stimulator ratio of 4:1 as described in Materials and Methods.
- b Peritoneal macrophage targets. Data are means of triplicates with spontaneous release subtracted.
- c Significantly more lysis than uninfected targets at the same effector:target ratio ( $P < 0.001$ ).
- d Significantly more lysis than uninfected targets at the same effector:target ratio ( $P < 0.01$ ).

TABLE 5

EFFECT OF DIFFERENT RESPONDER AND STIMULATOR CELLS ON THE  
RESPONSE<sup>a</sup> TO ECTROMELIA INFECTION

Experiment	Nature of responder cells	Nature of infected stimulator cells	% Yield of responder cells	Effector:target ratio	% <sup>51</sup> Cr release from targets <sup>b</sup>	
					Infected	Uninfected
1	Spleen	Macrophage	27%	15:1	63.4 <sup>c</sup>	0
				5:1	31.2 <sup>c</sup>	0
	Spleen + lymph node (7:1)	Macrophage	26%	15:1	65.1 <sup>c</sup>	11.1
				5:1	38.8 <sup>c</sup>	0
	Spleen	Spleen	17%	15:1	42.1 <sup>c</sup>	1.7
				5:1	18.1 <sup>c</sup>	0
	Spleen + lymph node (7:1)	Spleen	21%	15:1	31.7 <sup>c</sup>	7.4
				5:1	12.6 <sup>d</sup>	0
2	Spleen + lymph node (7:1)	Normal spleen	29%	5:1	52.3 <sup>c</sup>	14.9
	Spleen + lymph node (7:1)	Irradiated spleen (1000 R)	16%	5:1	27.8 <sup>c</sup>	9.7
	Spleen	Irradiated spleen (1000 R)	14%	5:1	7.2	8.2



TABLE 6

EFFECT OF RESPONDER:STIMULATOR RATIO ON PRIMARY RESPONSE  
IN VITRO OF BALB/c CELLS<sup>a</sup> TO ECTROMELIA INFECTION

Responder:stimulator ratio	% Yield of responder cells	% <sup>51</sup> Cr release from targets <sup>b</sup>	
		Infected	Uninfected
2:1	34%	33.4 <sup>c</sup>	9.7
4:1	26%	12.0 <sup>d</sup>	5.5
8:1	16%	0	2.8

a Responders were spleen + lymph node cells (7:1); stimulators were ectromelia-infected macrophages. Cultures were harvested at 5 days.

b Peritoneal macrophage targets at an effector:target ratio of 8:1. Data are means of triplicates with spontaneous release subtracted.

c Significantly more lysis than uninfected targets at the same effector:target ratio ( $P < 0.001$ ).

d Significantly more lysis than uninfected targets at the same effector:target ratio ( $P < 0.05$ ).

- a Responders were spleen + lymph node cells (7:1) and cultures were harvested after 5 days in 25 cm<sup>2</sup> flasks.
- b Macrophages were removed by adherence to carbonyl iron as described in Materials and Methods.
- c Macrophages were uninfected.
- d Peritoneal macrophage targets at effector:target ratios of 7.5:1 for spleen stimulators and 11:1 for macrophage stimulators. Data are means of triplicates with spontaneous release subtracted.
- e Significantly more lysis than uninfected targets at the same effector:target ratio ( $P < 0.001$ ).



TABLE 7

EFFECT OF STIMULATOR CELL TYPE AND RESPONDER:STIMULATOR RATIO ON PRIMARY RESPONSE

*IN VITRO* OF CBA/H CELLS<sup>a</sup> TO ECTROMELIA INFECTION

Stimulator cells	Responder macrophages <sup>b</sup> removed	6 x 10 <sup>5</sup> Macrophages <sup>c</sup> added per flask	Responder: stimulator ratio	% Yield of responder cells	% <sup>51</sup> Cr release from targets <sup>d</sup>	
					Infected	Uninfected
Spleen cells	No	No	3:2	20%	0	0.1
	No	Yes	3:2	31%	9.8	15.5
	Yes	No	3:2	18%	0	0.2
	Yes	Yes	3:2	28%	1.2	0
	Yes	Yes	3:1	20%	0	0
	Yes	Yes	6:1	12%	6.9	6.1
Macrophages	No	No	3:2	63%	30.5 <sup>e</sup>	3.6
	No	Yes	3:2	37%	38.6 <sup>e</sup>	6.9
	Yes	No	3:2	91%	30.8 <sup>e</sup>	4.2
	Yes	Yes	3:2	69%	35.4 <sup>e</sup>	1.4
	Yes	Yes	3:1	40%	26.2 <sup>e</sup>	0.9
	Yes	Yes	6:1	23%	7.6	1.5

TABLE 8

EFFECT OF MACROPHAGE DEPLETION ON THE PRIMARY RESPONSE *IN VITRO* OF CBA/H CELLS<sup>a</sup> TO  
ECTROMELIA INFECTION, AND EXPRESSION OF CYTOTOXIC ACTIVITY AGAINST MACROPHAGE  
AND L929 TARGET CELLS

Responder macrophages <sup>b</sup> removed	Responder: stimulator ratio	% Yield of responder cells	Effector:target ratio	% lysis of macrophages <sup>c</sup>		% lysis of L929 cells <sup>c</sup>	
				Infected	Uninfected	Infected	Uninfected
Yes	4:1	11%	5:1	1.4	4.1	n.d. <sup>f</sup>	n.d.
Yes	1:1	18%	5:1	9.4 <sup>e</sup>	0	n.d.	n.d.
No	4:1	24%	5:1	50.4 <sup>d</sup>	3.9	n.d.	n.d.
No	1:1	46%	5:1	58.4 <sup>d</sup>	2.4	22.9 <sup>e</sup>	11.1
No	1:1	46%	10:1	n.d.	n.d.	35.5 <sup>e</sup>	20.7
No	1:1	46%	20:1	n.d.	n.d.	52.9 <sup>e</sup>	39.5

a Responders were spleen and lymph node cells (7:1) and cultures were harvested at 5 days.

b Macrophages were removed as described in Materials and Methods.

c Data are mean % <sup>51</sup>Cr release from triplicates with spontaneous release subtracted.

d Significantly more lysis than on uninfected target cells of the same type at the same effector:target ratio ( $P < 0.001$ ).

e Significantly more lysis than on uninfected target cells of the same type at the same effector:target ratio ( $P < 0.02$ ).

f n.d. = not determined.



TABLE 9

COMPARISON OF CYTOTOXIC ACTIVITY OF C57BL/6 SPLEEN CELLS FROM  
 PRIMARY RESPONSE *IN VIVO*<sup>a</sup> AND PRIMARY AND SECONDARY  
 RESPONSES *IN VITRO*<sup>b</sup> TO ECTROMELIA VIRUS

Source of cytotoxic cells	Effector:target ratio	% <sup>51</sup> Cr release from targets <sup>c</sup>	
		Infected	Uninfected
Primary <i>in vivo</i>	4:1	33.8	6.6
	2:1	21.3	2.3
	1:1	18.3	2.4
Primary <i>in vitro</i>	8:1	43.5	9.0
	4:1	35.9	6.7
	2:1	15.4	3.6
	1:1	14.2	4.0
Secondary <i>in vitro</i>	1:1	41.3	2.9
	0.5:1	33.2	2.5

a Spleen cells from mice immunized intravenously with  $2 \times 10^4$  PFU of attenuated virus 5 days previously.

b *In vitro* responses used macrophage stimulators and responder: stimulator ratios of 4:1. Primary responders were spleen + lymph node cells (7:1), and secondary responders were spleen cells from mice primed *in vivo* (as above) 3 months previously.

c Peritoneal macrophage targets. Data given are means of triplicates with spontaneous release subtracted.

TABLE 10

EFFECT OF ANTI-THETA AND COMPLEMENT TREATMENT ON CYTOTOXIC ACTIVITY  
OF CELLS GENERATED IN THE PRIMARY RESPONSE *IN VITRO*<sup>a</sup> TO  
ECTROMELIA INFECTION

Treatment	Effector:target ratio	% <sup>51</sup> Cr release from macrophage targets <sup>b</sup>	
		Infected	Uninfected
Nil	15:1	48.4	15.7
Anti-theta and complement	15:1	1.5 <sup>c</sup>	0
Complement only	15:1	39.5	8.1

- a Responders were C57BL/6 spleen and lymph node cells (7:1) and stimulators were ectromelia-infected C57BL/6 spleen cells. Responder:stimulator ratio was 4:1 and cultures were harvested at 5 days.
- b Data given are means of triplicates with spontaneous release subtracted.
- c Significantly less lysis than untreated and complement-treated controls ( $P < 0.001$ ).



TABLE 11

H-2 RESTRICTION OF CYTOTOXIC CELLS GENERATED IN THE PRIMARY  
RESPONSE *IN VITRO*<sup>a</sup> TO ECTROMELIA INFECTION

Strain of primary responder and stimulator cells	% Specific lysis of infected macrophage targets <sup>b</sup>		
	B10.A(2R)	B10.A(5R)	B10.A
	kkkkkdddb	bbbkkdddb	kkkkkdddb
C57BL/6 bbbbbbbbb	33.3 <sup>c</sup>	29.4 <sup>c</sup>	2.2
B6.C-H-2 <sup>ba</sup> "ba"bbbbbbb	38.1 <sup>c</sup>	0.4	1.4

a Responders were spleen + lymph node cells (7:1) and stimulators were ectromelia virus-infected syngeneic spleen cells. Responder to stimulator ratio was 4:1 and cultures were harvested at 5 days. *H-2* maps of mice donating cells for primary response cultures and target macrophages are given and refer to *K*, *I-A*, *I-B*, *I-J*, *I-E*, *I-C*, *S*, *G* and *D* regions.

b Percentage specific lysis was calculated by first subtracting spontaneous release and then subtracting the small amount of release from uninfected control targets from release from infected targets. Data given were calculated from means of triplicates at an effector:target ratio of 5:1.

c Significantly more lysis than uninfected controls ( $P < 0.001$ ).

TABLE 12  
VIRUS SPECIFICITY OF C57BL/6 CYTOTOXIC T CELLS GENERATED IN  
PRIMARY RESPONSES<sup>a</sup> *IN VITRO*

Virus used to infect stimulators	Effector:target ratio	% <sup>51</sup> Cr release from C57BL/6 macrophage targets <sup>b</sup>		
		LCM-infected	Uninfected	Difference
LCM	10:1	33.6 <sup>c</sup>	6.5	27.1
Ectromelia	10:1	5.2	3.8	1.4
		Ectromelia- infected	Uninfected	Difference
LCM	5:1	17.9	21.2	-3.3
Ectromelia	5:1	44.8 <sup>c</sup>	12.3	32.5

a Responders were spleen + lymph node cells (7:1). LCM-infected stimulators were peritoneal macrophages and responder:stimulator ratio was 15:1. Ectromelia-infected stimulators were spleen cells and responder:stimulator ratio was 4:1. Cultures were harvested after 6 days at 37°C (LCM) or 39°C (ectromelia).

b Data given are means of triplicates with spontaneous release subtracted.

c Significantly higher lysis than uninfected controls or infected targets attacked by T cells from cultures containing the other virus (P < 0.001).



Significant portions of the amino acid sequences of H-2 and HLA antigens correspond, thus suggesting that the basic form of these antigens has been conserved through a considerable part of the evolution of vertebrates (Silver and Hood, 1976; Terhorst *et al.*, 1976). Despite this, some antigenic determinants borne on products of the major histocompatibility complex (MHC) are highly polymorphic, and the genes coding for these antigens are apparently mutating at an extraordinary rate (Selvick and Kohn, 1975). Thus, there seems to be evolutionary pressure to change certain antigenic features of MHC products, while conserving their basic form and function. Perhaps the search for T cell-mediated mechanisms allowing recovery from potentially lethal viral infections, such as ectromelia infection in mice (Kees and Blanden, 1976, see Chapter 2) is a

#### DIFFERENT H-2 REQUIREMENTS FOR TRIGGERING PRECURSOR T CELL INDUCTION OR LYSIS BY EFFECTOR T CELLS DEFINED BY THE BALB/C-H-2<sup>db</sup> MUTATION

infected target cells *in vitro* (reviewed by Doherty *et al.*, 1976a), and promote recovery from primary viral infection *in vivo* (Kees and Blanden, 1976) recognize H-2 antigens together with virus-specific antigens on infected cell surfaces (Doherty *et al.*, 1976a; Haff, 1976; Janeway *et al.*, 1976; Langman, 1977). The relevant genes in the H-2 complex map in either the K or the D region, but not in the I region (Kees and Blanden, 1976; Doherty *et al.*, 1976a; Blanden *et al.*, 1977; Blanden *et al.*, 1975; Zinkernagel *et al.*, 1976). Extensive experiments with five mutants of the H-2K<sup>b</sup> region and ectromelia, vaccinia, and lymphocytic choriomeningitis viruses (Kees and Blanden, 1976; Doherty *et al.*, 1976a; Zinkernagel, 1976; Blanden *et al.*, 1976; McKenzie *et al.*, 1977a) have shown that a single gene in H-2K<sup>b</sup>, probably a cistron coding for an H-2 polypeptide antigen (Brown and Nathansen, 1977) makes an essential, direct

## INTRODUCTION

Significant portions of the amino acid sequences of H-2 and HLA antigens correspond, thus suggesting that the basic form of these antigens has been conserved through a considerable part of the evolution of vertebrates (Silver and Hood, 1976; Terhorst *et al.*, 1976). Despite this, some antigenic determinants borne on products of the major histocompatibility complex (MHC) are highly polymorphic, and the genes coding for these antigens are apparently mutating at an extraordinary rate (Melvold and Kohn, 1975). Thus, there seems to be evolutionary pressure to change certain antigenic features of MHC products, while conserving their basic form and function. Perhaps the search for T cell-mediated mechanisms allowing recovery from potentially lethal viral infections, such as ectromelia infection in mice (Kees and Blanden, 1976, see Chapter 2) is a part of this evolutionary process. This idea is supported by findings that virus-immune cytotoxic T ( $T_c$ ) cells that specifically lyse virus-infected target cells *in vitro* (reviewed by Doherty *et al.*, 1976a), and promote recovery from primary viral infection *in vivo* (Kees and Blanden, 1976) recognize H-2 antigens together with virus-specific antigens on infected cell surfaces (Doherty *et al.*, 1976b; Raff, 1976; Janeway *et al.*, 1976; Langman, 1977). The relevant genes in the H-2 complex map in either the K or the D region, but not in the I region (Kees and Blanden, 1976; Doherty *et al.*, 1976a; Blanden *et al.*, 1977; Blanden *et al.*, 1975; Zinkernagel *et al.*, 1976). Extensive experiments with five mutants of the  $H-2K^b$  region and ectromelia, vaccinia, and lymphocytic choriomeningitis viruses (Kees and Blanden, 1976; Doherty *et al.*, 1976a; Zinkernagel, 1976; Blanden *et al.*, 1976; McKenzie *et al.*, 1977a) have shown that a single gene in  $H-2K^b$ , probably a cistron coding for an H-2 polypeptide antigen (Brown and Nathenson, 1977) makes an essential, direct



contribution to the antigenic patterns recognized by  $T_C$  cells specific for  $H-2K^b$  plus virus. Evidence from the  $H-2K^b$  mutants (McKenzie *et al.*, 1977a) and recent work with Sendai virus (Schrader and Edelman, 1977) suggests that H-2 antigens become physically associated with viral antigens in infected cell membranes, but the way this happens is unclear. Therefore, another important feature of H-2 antigens may be their capacity to form compound or interaction antigens by associating with viral gene products (Doherty *et al.*, 1976a; Zinkernagel and Doherty, 1974; Schrader *et al.*, 1975; Bevan, 1975).

The present experiments examine further the role of H-2 molecules in ectromelia virus-induced antigenic changes. We have used a new H-2 mutant, BALB/c- $H-2^{db}$ , that is unique among H-2 mutants characterized thus far (McKenzie *et al.*, 1977a; 1977b). It is carried in an inbred strain coisogenic with the parental strain (BALB/cKh). The mutation is of the loss type since BALB/c- $H-2^{db}$  reject BALB/cKh skin grafts, but not *vice versa*. Complementation studies have localized the mutation to the same locus in  $H-2D$  as B10.D2- $H-2^{da}$  (M504, see McKenzie *et al.*, 1977a; Egorov, 1967), a different gain-and-loss mutation. Serological analysis indicates that structure(s) identified by antibodies against the H-2.28 family of public specificities have been lost in BALB/c- $H-2^{db}$ , all other H-2 and Ia specificities being intact (McKenzie *et al.*, 1977b). Biochemical studies (Hansen *et al.*, 1977b) show that the  $H-2D^d$  region apparently codes for two similar proteins, each of about 45000 molecular weight. One of these bears serologically-defined private H-2 specificities; the other does not, but reacts with certain antibodies contained in anti-H-2.28 sera. It is the latter molecule which is not expressed in BALB/c- $H-2^{db}$ . This molecule has been designated D' by Hansen *et al.*, (1977b). We report here evidence which suggests that D' may be involved in a physical

association between  $H-2D^d$ -coded and viral antigens that is in turn required for efficient induction of precursors of  $T_C$  cells. In contrast,  $D'$  is apparently nonessential for target cell recognition and lysis by effector  $T_C$  cells.

## MATERIALS AND METHODS

### Mice

A.TH, A.TL, A.TFR2, A.TFR5, B10.A(2R), DBA/1, DBA/2, BALB/c, SJL/J, D2.GD, CBA/H, C3H.OH, C3H.OL, C57BL/6 and various  $F_1$  hybrids were bred at the John Curtin School. BALB/c- $H-2^{db}$  (mutant) and the coisogenic BALB/cKh (wild type) mice were bred at the Austin Hospital and shipped to the John Curtin School. All mice were used at 6-12 weeks of age.

### Virus

Stocks of attenuated (Hampstead egg) and virulent (Moscow) strains of ectromelia virus were grown, titrated, and stored as described in Chapter 2.

### Immunization

Mice were injected intravenously (iv.) with  $10^3$ - $10^4$  plaque-forming units (PFU) of attenuated virus; the  $T_C$  cell response was not affected by virus dose within this range (Blanden and Gardner, 1976). Spleens were taken for  $T_C$  cell assay on day 6, at the peak of the primary response (Gardner *et al.*, 1974; Blanden and Gardner, 1976), or after at least 3 weeks when they were used as a source of "memory" T cells for secondary responses *in vitro* (Gardner and Blanden, 1976; Pang and Blanden, 1976).

### Secondary T cell response *in vitro* to virus-infected cells

The basic methods used have been described in detail in Chapter 3. When the genotype of responder and stimulator cells was different (as in Table 4), infected macrophages were used as stimulators with procedures



designed to minimize escape of virus particles, or virus-specified antigens from the nominated stimulators into the responder population, which could in turn act as stimulators. Therefore, macrophage stimulators were infected in suspension with  $\gamma$ -irradiated Moscow virus (see Chapter 3) for 40 minutes at  $37^{\circ}\text{C}$  ( $10^8$  cells/ml; 1 PFU/cell). They were then washed three times in cold medium and dispensed into tissue culture flasks. The flasks were incubated at  $37^{\circ}\text{C}$  for 2-3 hours followed by vigorous washing (with warm medium) to remove nonadherent cells and residual virus before culture with memory T cells (responders). In addition, carbonyl iron-adherent (phagocytic) cells were removed from the responder population before culture. Responder:stimulator ratio was 8:1 and the cultures were held at  $39^{\circ}\text{C}$  (nonpermissive for viral replication) for 5 days before harvesting and assaying for  $T_c$  cell activity.

When spleen stimulator cells were used (as in Table 6), the cells were infected with  $\gamma$ -irradiated Moscow virus, washed three times with warm medium, left for 1.5-2 hours at  $37^{\circ}\text{C}$ , and then washed three more times before culturing for 5 days at  $39^{\circ}\text{C}$  with memory T cells at a responder:stimulator ratio of 10:1.

#### Response to minor histocompatibility (H) antigens

The method used was similar to that of Bevan (1975). DBA/2 mice were primed by i.v. injection of  $10^7$  spleen cells from either BALB/c or BALB/c-H-2<sup>db</sup>. After 14-16 days spleen cells from the primed mice were stimulated a second time *in vitro* by co-culturing for 5 days with irradiated BALB/c or mutant stimulator cells, and  $T_c$  cell activity against minor H antigens was then assayed against the appropriate macrophage target cells.

#### Cytotoxicity assay with macrophage target cells

The method has been described in detail in Chapter 3. Data given

have had spontaneous release subtracted and are the means of triplicates  $\pm$  standard errors (SE) of the mean (in some Tables, SE are omitted for the sake of clarity). Statistical significance was determined by Student's *t* test.

#### Anti-H-2 sera

The sera used were either obtained from the National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Md., or prepared and titrated as described previously (McKenzie and Snell, 1973).

(a) Anti-H-2.4: this serum was prepared as (B10.AKM x 129) $F_1$  anti-B10.A; it contains antibodies against the  $D^d$  private specificity (H-2.4) and cytotoxic titre against BALB/c cells was 1/256.

(b) Anti H-2.28: this was serum D28b obtained from NIAID and is described in the Catalogue of mouse alloantisera. It was prepared as (B10.Br x LP. RIII) $F_1$  anti-B10.A(2R) and contains antibodies against the H-2.28 family of public specificities. Cytotoxic titre against BALB/c cells was 1/256. This serum will be referred to as anti-H-2.28 for the sake of simplicity. It reacts with the molecule designated  $D'$  that is coded in the  $H-2^d$  region (Hansen *et al.*, 1977a; 1977b).

(c) Anti-H-2.31: this serum was prepared as (C57BL/6 x A) $F_1$  anti-B10.D2; it contains antibodies against the  $K^d$  private specificity H-2.31 and cytotoxic titre against BALB/c cells was 1/512.

#### Method for antisera-blocking of T cell-mediated lysis of macrophage targets

Target macrophages were infected with virus for 1 hour as described (in Chapter 3), then washed and left for 1 hour at  $37^\circ\text{C}$  to allow virus-induced antigens to be expressed. Medium was then removed, and anti-H-2 sera diluted one-half in complete medium containing 10% fetal calf serum were immediately added to each well of target cells in a volume of 0.1 ml and left for 30 minutes at  $37^\circ\text{C}$  before addition of  $T_c$  cells in a further



0.1 ml volume. Thus antisera were present at a one-quarter dilution throughout the 4 hours of the  $T_c$  cell assay. Data presented are representative of three experiments. It should be noted that blocking was demonstrable in this system only if a short assay time and potent secondary  $T_c$  cells were used.

## RESULTS

### Effect of the BALB/c-H-2<sup>db</sup> mutation on virus-specific T cell-mediated lysis

BALB/c-H-2<sup>db</sup>, BALB/cKh, and a number of other strains with recombinant H-2 haplotypes were immunized with ectromelia virus. Virus-immune  $T_c$  cells from A.TH, A.TFR5, A.TL, and A.TFR2 mice, which share only the D region with BALB/cKh (wild type) or BALB/c-H-2<sup>db</sup> (mutant) lysed infected targets of either mutant or wild type with similar efficiency (Table 1). In contrast, virus-immune  $T_c$  cells from BALB/c-H-2<sup>db</sup> mice gave little or no significant lysis of infected A.TH or A.TFR2 targets (Tables 1 and 2), but did kill infected self or infected BALB/cKh targets, presumably through H-2K<sup>d</sup> sharing (Table 1). Thus the mutation did not seem to directly affect the virus-induced antigenic change(s), dependent upon the H-2D<sup>d</sup> region, which are required for recognition and lysis by virus-immune  $T_c$  cells. But these same antigenic change(s) apparently induced only a very weak  $T_c$  cell response in BALB/c-H-2<sup>db</sup> mice.

F<sub>1</sub> hybrids between BALB/c-H-2<sup>db</sup> and B10.A(2R), DBA/1, or SJL were also tested; these last three strains all possess a gene for members of the H-2.28 family of specificities (McKenzie *et al.*, 1977b). After immunization with virus, the F<sub>1</sub> hybrids gave little or no significant  $T_c$  cell-mediated lysis of infected A.TH or A.TFR5 target cells (Table 2). Thus, introducing genes for other versions of H-2.28 was insufficient to overcome the effect of the BALB/c-H-2<sup>db</sup> mutation.

Capacity of BALB/c-H-2<sup>db</sup> mice to respond to virus-induced antigenic changes associated with their own H-2D region

Although BALB/c-H-2<sup>db</sup> mice generated few virus-immune T<sub>C</sub> cells that recognized wild-type H-2D<sup>d</sup> determinants plus virus-specific antigen, they may have responded to virus-induced changes specific for mutant H-2D antigenic patterns. This was tested by taking pools of BALB/c-H-2<sup>db</sup> and D2.GD virus-immune T<sub>C</sub> cells and assaying them for a short time at various effector:target ratios against BALB/c-H-2<sup>db</sup> and C3H.OH macrophage targets. Lysis of infected C3H.OH targets by these two T<sub>C</sub> cell pools was similar at 10:1 (BALB/c-H-2<sup>db</sup>) and 5:1 (D2.GD) (Table 3). This lysis would be caused by T<sub>C</sub> cell clones recognizing virus-specific antigenic changes associated with H-2K<sup>d</sup> (Blanden *et al.*, 1975; Zinkernagel *et al.*, 1976). If the BALB/c-H-2<sup>db</sup> T<sub>C</sub> cell pool contained additional clones recognizing virus-induced changes specific for the mutant H-2D region, then it should cause more lysis of infected BALB/c-H-2<sup>db</sup> targets than the D2.GD T<sub>C</sub> cell population. This was not the case; lysis was again similar (Table 3), thus suggesting a poor response to mutant H-2D region-associated, virus-induced antigenic patterns. This is not definitive, however, since in our hands, effector:target dose-response relationships are not reproducible with macrophage target cells (Blanden *et al.*, 1976). Attempts to resolve the issue with the cold target competition approach (Zinkernagel, 1976) were also unsuccessful.

Capacity of infected BALB/c-H-2<sup>db</sup> macrophages to stimulate T<sub>C</sub> cell responses

While infected BALB/c-H-2<sup>db</sup> macrophages display virus-induced antigens recognizable by virus-immune T<sub>C</sub> cells from wild-type H-2D<sup>d</sup> mice (Table 1), the T<sub>C</sub> cell response against these antigens in BALB/c-H-2<sup>db</sup> mice is weak or absent. The ability of infected BALB/c-H-2<sup>db</sup> macrophages to stimulate wild-type H-2D<sup>d</sup> T<sub>C</sub> cells was therefore tested. Memory T<sub>C</sub>



cells from virus-primed (CBA/H x BALB/c)  $F_1$  mice were used as responders, to detect any escape of virus-specific antigen from the nominated stimulators into some responder cells.

BALB/c and BALB/c-H-2<sup>db</sup>-infected macrophages stimulated similar responses against infected C3H.OL targets, where lysis is caused by  $T_C$  cells recognizing virus-specific plus H-2K<sup>d</sup> antigens (Table 4). But BALB/c-H-2<sup>db</sup> stimulators were significantly inferior to BALB/c in stimulating  $T_C$  cells that recognize virus-specific plus H-2D<sup>d</sup> antigens on infected A.TH targets (Table 4, Experiment 1). Minor escape of virus-specific antigen into the (CBA/H x BALB/c) $F_1$  responder cells might have occurred, since slight lysis of infected CBA/H targets was seen (Table 4, Experiment 1). A second, similar, experiment with (C57BL/6 x BALB/c) $F_1$  responders gave similar results (Table 4, Experiment 2) except that no escape of antigen into responder cells was detected. Thus, it seems that infected BALB/c-H-2<sup>db</sup> cells are significantly inferior to wild type in terms of stimulating a  $T_C$  cell response against virus-specific plus H-2D<sup>d</sup>-coded determinants. These data suggest the possibility that the physical relationships between virus-specific and H-2D<sup>d</sup> molecules that may be required for efficient induction of virus-immune  $T_C$  cells could be impaired by the absence of D' (Hansen *et al.*, 1977b) from the surfaces of BALB/c-H-2<sup>db</sup> cells.

#### Capacity of uninfected BALB/c-H-2<sup>db</sup> cells to stimulate $T_C$ cell responses to minor H antigens

The  $T_C$  cell response to minor H antigens exhibits H-2 restriction (Bevan, 1975). Thus it offers a means to test whether the stimulation defect of BALB/c-H-2<sup>db</sup> reflects an intrinsic failure of *D* region-coded molecule(s) to provide the antigenic configurations needed for  $T_C$  cell stimulation, or whether the defect lies in a failure of BALB/c-H-2<sup>db</sup>

molecule(s) to associate adequately with virus-specific antigen.

Spleen cells from DBA/2 ( $H-2^d$ ) mice primed with either BALB/c or BALB/c- $H-2^{db}$  cells, were stimulated a second time *in vitro* with irradiated BALB/c or BALB/c- $H-2^{db}$  cells, respectively.  $T_c$  cell activity was then assayed against C3H.OL ( $K^d, D^k$ ) and A.TL ( $K^s, D^d$ ) macrophage targets that would display minor H antigens shared with the BALB/c background in association with either  $H-2K^d$  (C3H.OL) or  $H-2D^d$  (A.TL) determinants. At a given effector:target ratio, C3H.OL and A.TL targets were lysed to a similar extent, regardless of whether stimulation was from BALB/c or BALB/c- $H-2^{db}$  cells (Table 5). CBA/H ( $K^k, D^k$ ) targets were not lysed, and SJL/J ( $K^s, D^s$ ) targets were lysed slightly. These data confirm that the  $T_c$  cell response to minor H antigens is H-2 restricted to some extent (Bevan, 1975) and also indicate that little, if any, of the lysis of C3H.OL and A.TL targets is due to nonspecifically activated  $T_c$  cells that recognize  $H-2K$  or  $H-2D$  antigens alone. Apparently, therefore, minor H antigens are as immunogenic in association with  $H-2D$ -coded antigens of BALB/c- $H-2^{db}$  as they are with wild type. Similar results have been obtained by using trinitrophenyl-modified cells (data not shown). These results reinforce the possibility that the defect in the  $T_c$  cell response to ectromelia infection in BALB/c- $H-2^{db}$  mice may be caused by inefficient physical association between mutant  $H-2D$ -coded and virus-specific molecules.

#### Specific blocking of $T_c$ cell-mediated lysis with anti- $H-2K$ and anti- $H-2D$ sera

Specific anti- $H-2$  sera against the determinants  $H-2.4$  ( $D^d$ , private)  $H-2.28$  ( $D^d$ , public, borne on  $D^d$ ) and  $H-2.31$  ( $K^d$ , private) were used. Virus-infected BALB/c or BALB/c- $H-2^{db}$  macrophages were attacked by two separate subsets of virus-immune  $T_c$  cells, one subset from D2.GD mice that recognized virus-induced plus  $H-2K^d$  antigens and the second subset from



A.TH mice that recognized virus-induced plus H-2D<sup>d</sup> antigens (Blanden *et al.*, 1975; Zinkernagel *et al.*, 1976). Lysis of both BALB/c and BALB/c-H-2<sup>db</sup> targets by D2.GD T<sub>C</sub> cells was blocked significantly more by anti-H-2.31 than by anti-H-2.4 and anti-H-2.28 (Table 6). Conversely, lysis of BALB/c targets by A.TH T<sub>C</sub> cells was blocked more by anti-H-2.4 and anti-H-2.28 than by anti-H-2.31 (Table 6). Most importantly, anti-H-2.28 failed to block lysis of BALB/c-H-2<sup>db</sup> targets by A.TH T<sub>C</sub> cells while anti-H-2.4 was active (Table 6). This is consistent with the loss of D' from the surface of mutant BALB/c-H-2<sup>db</sup> cells (McKenzie *et al.*, 1977b; Hansen *et al.*, 1977b), and also suggests that the blocking activity of the sera was directed at target cells, not T<sub>C</sub> cells, since A.TH T<sub>C</sub> cells possess D'.

The specificity of blocking (Table 6) indicated that T<sub>C</sub> cells recognized virus-induced antigenic changes which incorporated, or were physically close to, either H-2K or H-2D molecules bearing serologically defined determinants. In the case of H-2D<sup>d</sup> antigens on BALB/c cells, a molecule with private determinants (H-2.4) and the D' molecule were apparently both involved.

#### DISCUSSION

The present experiments showed that ectromelia-infected cells of the BALB/c-H-2<sup>db</sup> line display virus-induced antigenic patterns associated with the H-2D region, but BALB/c-H-2<sup>db</sup> T<sub>C</sub> cell precursors seem to respond weakly, if at all, to these antigenic patterns. BALB/c-H-2<sup>db</sup> is a loss mutation apparently in a gene required for production or expression on cell membranes of a 45000 molecular weight molecule, designated D' by Hansen *et al.* (1977b), that bears certain H-2 public specificities (McKenzie *et al.*, 1977b). No other H-2 or Ia antigens seem to be affected (McKenzie *et al.*, 1977b). Hypotheses to account for the defective response to ectromelia virus infection were considered in this context.

First, has there been deletion or suppression of a gene for a  $T_C$  cell receptor needed for recognition of H-2D<sup>d</sup> antigenic determinants on virus-infected cells? This seems unlikely, since variable regions of  $T_C$  cell receptors defined thus far are not coded by MHC-linked genes (Raff, 1976; Haemmerling *et al.*, 1976).  $T_C$  cell recognition structures physically associated with D' also seem to be disqualified, since anti-H-2 antibody blocked  $T_C$  cell-mediated lysis by binding to target cells, not to  $T_C$  cells (Table 6).

Second, does the absence of the D' molecule in BALB/c-H-2<sup>db</sup> expose other H-2D antigenic determinants on infected cells, recognizable by  $T_C$  cells, that are not normally exposed on infected wild-type cells? These exposed determinants, plus virus-specific determinants, could then be the predominant inducers of virus-immune  $T_C$  cells in BALB/c-H-2<sup>db</sup> mice. There are several points of evidence against this hypothesis, though none of them is definitive. It seems unlikely that new H-2D determinants unique to BALB/c-H-2<sup>db</sup> should be present on infected cells, in view of the findings that uninfected BALB/c-H-2<sup>db</sup> cells show no such determinants on the basis of serological or skin graft criteria (McKenzie *et al.*, 1977b). Also, this hypothesis predicts that BALB/c-H-2<sup>db</sup> mice should generate a normal level of virus-immune  $T_C$  cell response against their own H-2D determinants plus viral antigen; such a response is not evident (Table 3). Finally, and most importantly, it does not account for the demonstrated defect in the capacity of infected BALB/c-H-2<sup>db</sup> macrophages to stimulate virus-immune memory  $T_C$  cells that recognize H-2D<sup>d</sup> determinants (Table 4). This is despite observations that such infected macrophages are lysed to the same extent as wild-type targets by  $T_C$  cells recognizing wild-type H-2D antigens plus virus-specific determinants, and that BALB/c-H-2<sup>db</sup>-infected macrophages are not defective in stimulating virus-immune  $T_C$  cells that recognize



H-2K<sup>d</sup> determinants.

The possibility exists, therefore, that the minimum requirements for precursor T<sub>C</sub> cell induction (including memory T<sub>C</sub> cells) are more rigorous than those for the process of antigen recognition by effector T<sub>C</sub> cells that leads to lysis of infected targets. It seems that the presence of the D' molecule, plus the molecule bearing H-2D<sup>d</sup>-coded private determinant(s), plus virus-specific antigen on the infected cell surface are all required for precursor T<sub>C</sub> cell induction, but that D' is nonessential for effector T<sub>C</sub> cell recognition and lysis. When tested for capacity to stimulate T<sub>C</sub> cell responses against minor H antigens or trinitrophenyl in association with H-2D<sup>d</sup>-coded determinants, BALB/c-H-2<sup>db</sup> cells were as efficient as BALB/c cells. Thus, there seems to be no intrinsic defect in the molecule bearing H-2D<sup>d</sup>-coded private determinants in terms of T<sub>C</sub> cell induction. This suggests that the poor inductive capability of infected BALB/c-H-2<sup>db</sup> cells with respect to virus-specific T<sub>C</sub> cells may reside in the manner in which the viral and the two H-2D-coded molecules physically associate in infected cell membranes. The D' molecule may play a crucial role in this association. That a close physical relationship does exist between both of the H-2D<sup>d</sup>-coded molecules and virus-specific determinants on the surface of infected cells was shown by the specificity of blocking of virus-immune T<sub>C</sub> cell-mediated lysis by monospecific anti-H-2 sera directed against both private and public determinants (Table 6).

The foregoing evidence, taken together with findings from the H-2K<sup>b</sup> mutations (McKenzie *et al.*, 1977a) suggests that there are two features of H-2 molecules that are pertinent to the H-2 restriction phenomenon. The first is a structure bearing antigenic determinant(s) that are recognized by receptors on T<sub>C</sub> cells; such determinant(s) are affected by the H-2K<sup>b</sup> mutants (McKenzie *et al.*, 1977a), but do not seem to be affected in

BALB/c- $H-2^{db}$ . The second may be a structure required for physical association between the H-2 determinant(s) and a virus-specified antigen molecule; such association seems necessary for efficient induction of virus-specific  $T_C$  cells. This class of structure is unaffected by the  $H-2K^b$  mutations (McKenzie *et al.*, 1977a) but appears to be affected in BALB/c- $H-2^{db}$ . In the case of products of the  $H-2D^d$  region, this structure is apparently embodied in a second molecule (D') that does not bear private H-2 specificities, but can be identified by antibodies in anti-H-2.28 sera (McKenzie *et al.*, 1977b; Hansen *et al.*, 1977b; Démant *et al.*, 1975; Lemonnier *et al.*, 1975). The unusual distribution of the genes for the H-2.28 and H-2.1 families of public specificities among murine  $H-2K$  and  $H-2D$  regions discussed by Snell *et al.* (1973; 1974) and Démant *et al.* (1975) also deserves comment in this connection. It seems that the H-2.28 and H-2.1 specificities are never both coded in the same  $H-2K$  or  $H-2D$  region, but either one or the other is present (Démant *et al.*, 1975; Snell *et al.*, 1973; 1974). This raises the possibility that an H-2.1-bearing molecule serves a similar function in other mouse strains to that ascribed to D' in the present experiments with BALB/c. To test this possibility may require appropriate loss mutations.

#### SUMMARY

The  $T_C$  cell response to ectromelia virus infection was studied in BALB/c- $H-2^{db}$  mice which carry a loss mutation in the  $H-2D$  region that results in the absence from cell surfaces of a molecule (D') bearing certain public H-2 specificities. When infected, these mice showed a poor response of  $T_C$  cells that recognize  $H-2D^d$  plus virus-specific determinants on infected macrophage targets, but gave a normal response to  $H-2K^d$  plus virus-specific antigens. However, their own infected macrophages do display wild-type antigenic patterns involving virus and  $H-2D^d$



since they were killed as efficiently as wild-type (BALB/c, H-2<sup>d</sup>)-infected cells by T<sub>c</sub> cells specific only for H-2D<sup>d</sup> plus viral antigens. When tested *in vitro*, infected BALB/c-H-2<sup>db</sup> cells stimulated a poor T<sub>c</sub> cell response to H-2D plus virus-specific antigens, but stimulated a normal response (in comparison with infected BALB/c macrophages) to H-2K<sup>d</sup> plus viral antigens. Uninfected BALB/c-H-2<sup>db</sup> cells stimulated a normal T<sub>c</sub> cell response to minor H antigens or trinitrophenyl in association with H-2D<sup>d</sup>, thus suggesting that the defective response to infection may reside in a failure of the relevant H-2D<sup>d</sup> antigens of mutant cells to physically associate with viral antigens. Close association of viral and H-2D-coded molecules was also suggested by ability of specific anti-H-2K or -H-2D to partially block T<sub>c</sub> cell-mediated lysis of infected targets.

These results were interpreted to mean that H-2D<sup>d</sup>-dependent, virus-immune T<sub>c</sub> cells recognized an antigenic pattern consisting of virus-specific and H-2D<sup>d</sup> determinants with the latter borne on an H-2D molecule carrying serologically-defined H-2D<sup>d</sup> private specificities. A second H-2D<sup>d</sup>-coded molecule (D') was not required for recognition and lysis by activated T<sub>c</sub> cells, but was apparently necessary for efficient stimulation of precursor T<sub>c</sub> cells, perhaps by promoting appropriate physical association of viral and H-2D<sup>d</sup> molecules.

## REFERENCES

- Bevan, M. J. 1975. Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. *Nature (London)* 256:419.
- Blanden, R. V., Doherty, P. C., Dunlop, M. B. C., Gardner, I. D., Zinkernagel, R. M. and David, C. S. 1975. Genes required for cytotoxicity against virus infected target cells in K and D regions of H-2 complex. *Nature (London)* 254:269.
- Blanden, R. V. and Gardner, I. D. 1976. The cell-mediated immune response to ectromelia virus infection. I. Kinetics and characteristics of the primary effector T cell response *in vivo*. *Cell.Immunol.* 22:271.
- Blanden, R. V., Dunlop, M. B. C., Doherty, P. C., Kohn, H. I. and McKenzie, I. F. C. 1976. Effects of four H-2K mutations on virus-induced antigens recognized by cytotoxic T cells. *Immunogenetics* 3:541.
- Blanden, R. V., Pang, T. E. and Dunlop, M. B. C. 1977. T cell recognition of virus-infected cells, in: *Virus infection and the cell surface, Cell Surface Reviews, Vol. 2*, G. Poste and G. L. Nicholson, eds., North Holland Publishing Company, Amsterdam, p. 249.
- Brown, J. L. and Nathenson, S. G. 1977. Structural differences between parent and mutant H-2K glycoproteins from two H-2K gene mutants: B6.C-H-2<sup>ba</sup> (Hz1) and B6-H-2<sup>bd</sup> (M505). *J.Immunol.* 118:98.
- Démant, P., Snell, G. D., Hess, M., Lemonnier, F., Neauporte-Sautes, C. and Kourilsky, F. 1975. Separate and polymorphic genes controlling two types of polypeptide chains bearing the H-2 private and public specificities. *J.Immunogenetics* 2:263.
- Doherty, P. C., Blanden, R. V. and Zinkernagel, R. M. 1976a. Specificity of virus-immune effector T cells for H-2K or H-2D compatible interactions: implications for H-antigen diversity. *Transplant.Rev.* 26:89.
- Doherty, P. C., Gotze, D., Trinchieri, G. and Zinkernagel, R. M. 1976b. Models for recognition of virally-modified cells by immune thymus-derived lymphocytes. *Immunogenetics* 3:517.
- Egorov, I. K. 1967. A mutation of the histocompatibility-2 locus in the mouse. *Genetika* 3:136.
- Gardner, I., Bown, N. A. and Blanden, R. V. 1974. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. *Eur.J.Immunol.* 4:63.
- Gardner, I. D. and Blanden, R. V. 1976. The cell-mediated immune response to ectromelia virus infection. II. Secondary response *in vitro* and kinetics of memory T cell production *in vivo*. *Cell.Immunol.* 22:283.



- Haemmerling, G. J., Black, S. J., Eichmann, C. B. K. and Rajewsky, K. 1976. Idiotypic analysis of lymphocytes *in vitro*. II. Genetic control of T-helper cell responsiveness to antiidiotypic antibody. *J.Exp.Med.* 143:861.
- Hansen, T. H., Cullen, S. E. and Sachs, D. H. 1977a. Immunochemical evidence for an additional H-2 region closely-linked to H-2D. *J.Exp.Med.* 145:438.
- Hansen, T. H., Cullen, S. E., Melvold, R., Kohn, H., Flaherty, L. and Sachs, D. H. 1977b. Mutation in a new H-2 associated histocompatibility gene closely-linked to H-2D. *J.Exp.Med.* 145:1550.
- Janeway, C. A., Wigzell, H. and Binz, H. 1976. Two different V<sub>H</sub> gene products make up the T cell receptors. *Scand.J.Immunol.* 5:993.
- Kees, U. and Blanden, R. V. 1976. A single genetic element in H-2K affects mouse T cell antiviral function in poxvirus infection. *J.Exp.Med.* 143:450.
- Langman, R. E. 1977. Cell-mediated immunity and the major histocompatibility complex. *Rev.Phys.Biochem.Pharm.* 81:1.
- Lemonnier, F., Neauporte-Sautes, C., Kourilsky, F. M. and Démant, P. 1975. Relationships between private and public H-2 specificities on the cell surface. *Immunogenetics* 2:517.
- McKenzie, I. F. C. and Snell, G. D. 1973. Comparative immunogenicity and enhanceability of individual H-2K and H-2D specificities of murine histocompatibility. *J.Exp.Med.* 138:250.
- McKenzie, I. F. C., Pang, T. and Blanden, R. V. 1977a. The use of H-2 mutants as models for the study of T cell activation. *Immunol.Rev.* 35:181.
- McKenzie, I. F. C., Morgan, G. M., Melvold, R. W. and Kohn, H. K. 1977b. BALB/c-H-2<sup>db</sup>: a new H-2 mutant in BALB/cKh that identifies a locus associated with the D region. *Immunogenetics* 4:333.
- Melvold, R. W. and Kohn, H. I. 1975. Histocompatibility gene mutation rates: H-2 and non-H-2. *Mutat.Res.* 27:415.
- Pang, T. and Blanden, R. V. 1976. The cell-mediated immune response to ectromelia virus infection. Secondary response *in vitro*: specificity, nature of effector and responder cells and the requirements for induction of antigenic changes in stimulator cells. *Aust.J. Exp.Biol.Med.Sci.* 54:253.
- Raff, M. 1976. T cell recognition at Cold Spring Harbor. *Nature(London)* 263:10.
- Schrader, J. W., Cunningham, B. A. and Edelman, G. M. 1975. Functional interactions of viral and histocompatibility antigens at tumor cell surfaces. *Proc.Natl.Acad.Sci. USA* 72:5068.

- Schrader, J. W. and Edelman, G. M. 1977. Joint recognition by cytotoxic T cells of inactivated Sendai virus and products of the major histocompatibility complex. *J.Exp.Med.* 145:523.
- Silver, J. and Hood, L. 1976. Structure and evolution of transplantation antigens: partial amino acid sequences of H-2K and H-2D allo-antigens. *Proc.Natl.Acad.Sci. USA* 73:599.
- Snell, G. D., Cherry, M. and Démant, P. 1973. H-2: Its structure and similarity to HL-A. *Transplant. Rev.* 15:3.
- Snell, G. D., Démant, P. and Cherry, M. 1974. Haemagglutination and cytotoxic studies of H-2. V. The anti-27, 28, 29 family of antibodies. *Folia Biol. (Prague)* 20:145.
- Terhorst, C., Parham, P., Mann, D. L. and Strominger, J. L. 1976. Structure of the HLA antigens: amino-acid and carbohydrate compositions and NH<sub>2</sub>-terminal sequences of four antigen preparations. *Proc. Natl.Acad.Sci. USA* 73:910.
- Zinkernagel, R. M. and Doherty, P. C. 1974. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature(London)* 251:547.
- Zinkernagel, R. M. 1976. H-2 compatibility requirement for virus-specific T cell-mediated cytotoxicity. The H-2K structure involved is coded for by a single cistron defined by B6 (H21) and B6 (H2170) H-2K<sup>b</sup> mutant mice. *J.Exp.Med.* 143:437.
- Zinkernagel, R. M., Dunlop, M. B. C., Blanden, R. V., Doherty, P. C. and Shreffler, D. C. 1976. H-2 compatibility requirement for virus-specific T cell-mediated cytotoxicity. Evaluation of the role of H-2I region and non-H-2 genes in regulating immune responses. *J.Exp.Med.* 144:519.



TABLE 1  
EFFECT OF THE BALB/c-H-2<sup>db</sup> MUTATION ON T CELL-MEDIATED LYSIS  
OF VIRUS-INFECTED MACROPHAGES

Experiment	Donors of virus-immune T cells <sup>a</sup>							% Specific lysis of infected targets <sup>b</sup>			
	Strain	H-2 map						A.TH	A.TFR5	BALB/c-H-2 <sup>db</sup>	BALB/cKh
		K	I-A	I-B	I-C	S	D				
1	A.TH	s	s	s	s	s	d	24.0	13.5	32.7	20.4
	A.TFR5	f	f	f	f	k	d	35.6	34.0	33.0	41.2
	BALB/c-H-2 <sup>db</sup>	d	d	d	d	d	db	3.2 <sup>c</sup>	0 <sup>c</sup>	47.7	43.6
	BALB/cKh	d	d	d	d	d	d	36.8	31.3	46.7	42.1
								A.TL	A.TFR5	BALB/c-H-2 <sup>db</sup>	BALB/cKh
2	A.TL	s	k	k	k	k	d	41.3	27.8	24.7	34.7
	A.TFR2	f	f	f	f	s	d	36.0	33.9	42.9	35.2

- a T cells obtained from pools of two spleens at the peak of the primary response *in vivo* 6 days after i.v. immunization with attenuated virus.
- b Percent <sup>51</sup>Cr release from ectromelia-infected targets over a 16-hour period with spontaneous release and release from uninfected controls subtracted. Effector:target ratio was 30:1. Means of triplicates are given. SE of means were never larger than 2.9 (Experiment 1) and 2.5 (Experiment 2).
- c Not significantly greater lysis than uninfected control targets. All other effector:infected target combinations gave significant lysis (P < 0.01).

- a T cells obtained from pools of two spleens at the peak of the primary response *in vivo* 6 days after i.v. immunization with attenuated virus.
- b As for Table 1 except that SE of means were never larger than 3.3 (Experiment 1) and 3.1 (Experiment 2).
- c All cases where BALB/c-H-2<sup>db</sup> or F<sub>1</sub> T cells were placed on infected targets from recombinants (A.TH and A.TFR5) sharing only D region with BALB/c gave significantly less lysis ( $P < 0.001$ ) than where the same T cells were placed on infected BALB/cKh targets.
- d Significantly more lysis than uninfected control targets ( $P < 0.05$ ).
- e n.d. = not determined.



TABLE 2

EFFECT OF THE BALB/c-H-2<sup>db</sup> MUTATION IN HETEROZYGOTES CONTAINING A GENE FOR H-2.28

Experiment	Donors of virus-immune T cells <sup>a</sup>							% Specific lysis of infected targets <sup>b</sup>			
	Strain	H-2 map						A.TH	A.TFR5	BALB/cKH (dddddd)	C57BL/6 (bbbbbb)
		K	I-A	I-B	I-C	S	D				
1	A.TFR5	f	f	f	f	k	d	38.8	43.8	39.8	0
	BALB/c-H-2 <sup>db</sup>	d	d	d	d	d	db	7.4 <sup>cd</sup>	11.8 <sup>cd</sup>	39.9	1.3
	B10.A(2R)	k	k	k	d	d	b	0.5	0	0	44.9
	(B10.A(2R) x BALB/c-H-2 <sup>db</sup> )F <sub>1</sub>							0 <sup>c</sup>	0 <sup>c</sup>	44.0	28.5
								A.TFR5	BALB/cKh	DBA/1	SJL/J
2	A.TFR5	f	f	f	f	k	d	35.6	35.4	n.d. <sup>e</sup>	n.d.
	DBA/1	q	q	q	q	q	q	0	0	39.2	n.d.
	(DBA/1 x BALB/c-H-2 <sup>db</sup> )F <sub>1</sub>							10.8 <sup>cd</sup>	38.1	n.d.	n.d.
	SJL/J	s	s	s	s	s	s	0	0	n.d.	26.9
	(SJL/J x BALB/c-H-2 <sup>db</sup> )F <sub>1</sub>							9.0 <sup>cd</sup>	27.6	n.d.	n.d.

TABLE 3

RELATIVE EFFICIENCY OF LYSIS OF INFECTED SELF OR INFECTED H-2K-COMPATIBLE TARGETS BY  
BALB/c-H-2<sup>db</sup> VIRUS-IMMUNE T CELLS

Donors of virus-immune T cells <sup>a</sup>	Effector:target ratio	% Specific lysis of macrophage targets <sup>c</sup>					
		BALB/c-H-2 <sup>db</sup> (ddddd"db") <sup>b</sup>		C3H.OH (dddddK)		A.TH (sssssd)	
		Infected	Uninfected	Infected	Uninfected	Infected	Uninfected
BALB/c-H-2 <sup>db</sup> (ddddd"db")	10:1	26.0 ± 1.7	2.5 ± 0.1	21.6 ± 3.1	0	2.5 ± 2.1	1.2 ± 0.5
D2.GD (ddbbbbb)	5:1	29.6 ± 1.7	0	24.4 ± 2.6	0	0	0

a As for Table 1.

b H-2 map regions as for Table 1.

c Assay run for 4 hours. Data given are means of triplicates ± SE of the mean with spontaneous release subtracted.



- a Cytotoxic T cells were generated in secondary responses *in vitro* (see Materials and Methods).
- b Number of viable responder cells after 5 days *in vitro* expressed as a percentage of the original responder number set up.
- c H-2 map regions as for Table 1.
- d Assay run for 6 hours at effector:target ratios indicated; data given are means of triplicates  $\pm$  SE of the mean; otherwise as for Table 1.
- e Significantly less lysis than that caused by T cells stimulated by infected BALB/c macrophages and assayed on infected A.TH or A.TL targets at the same effector:target ratio ( $P < 0.001$ ).

TABLE 4

CAPACITY OF INFECTED BALB/c-H-2<sup>db</sup> MACROPHAGES TO STIMULATE CYTOTOXIC T CELL CLONES  
THAT RECOGNIZE VIRUS-INDUCED ANTIGENIC PATTERNS ASSOCIATED WITH EITHER H-2K or H-2D

Experiment	Responder T cells <sup>a</sup>	Infected macrophage stimulator cells	Yield <sup>b</sup>	Killer target ratio	% Specific lysis of infected targets <sup>d</sup>		
					C3H.OL (ddddkk) <sup>c</sup>	A.TH (sssssd)	CBA/H (kkkkkk)
			%				
1	(CBA/H x BALB/c)F <sub>1</sub>	BALB/c (ddddd)	32	5:1	31.6 ± 2.8	19.7 ± 1.3	4.7 ± 1.5
				2:1	26.2 ± 0.9	16.8 ± 1.9	2.1 ± 1.3
	(CBA/H x BALB/c)F <sub>1</sub>	BALB/c-H-2 <sup>db</sup> (ddddd"db")	32	5:1	35.1 ± 6.2	6.9 ± 0.9 <sup>e</sup>	4.9 ± 1.6
				2:1	23.7 ± 2.2	3.5 ± 1.7 <sup>e</sup>	0
					C3H.OL (ddddkk)	A.TL (skkkkd)	C57BL/6 (bbbbbb)
2	(C57BL/6 x BALB/c)F <sub>1</sub>	BALB/c (ddddd)	44	8:1	27.1 ± 1.1	23.5 ± 1.0	0
				2:1	15.7 ± 1.3	13.0 ± 1.7	0
	(C57BL/6 x BALB/c)F <sub>1</sub>	BALB/c-H-2 <sup>db</sup> (ddddd"db")	38	8:1	26.6 ± 1.6	10.1 ± 0.9 <sup>e</sup>	0
				2:1	27.8 ± 1.8	0.7 ± 0.5 <sup>e</sup>	0



TABLE 5

CYTOTOXIC T CELL RESPONSE TO MINOR H ANTIGENS STIMULATED BY BALB/c AND  
BALB/c-H-2<sup>db</sup> CELLS

Responder <sup>a</sup> T cells	Irradiated stimulator cells	Yield <sup>b</sup>	Effector:target ratio	% Specific lysis of macrophage targets <sup>d</sup>			
				C3H.OL (dddddkk) <sup>c</sup>	A.TL (skkkkd)	CBA.H (kkkkkk)	SJL/J (ssssss)
		%					
DBA/2 (ddddd)	BALB/c (ddddd)	51	7.5:1	30.9 ± 2.6	32.0 ± 4.4	0	15.0 ± 0.8
			2.5:1	10.5 ± 2.3	13.0 ± 3.0	0	6.1 ± 4.6
DBA/2 (ddddd)	BALB/c-H-2 <sup>db</sup> (ddddd"db")	90	2.5:1	35.5 ± 1.9	32.2 ± 2.0	0	10.8 ± 1.6
			0.8:1	21.8 ± 2.4	19.5 ± 1.8	0	3.7 ± 3.1

a Cytotoxic T cells were generated in secondary responses to minor H antigens *in vitro* (see Materials and Methods).

b As for Table 4.

c H-2 regions as for Table 1.

d Assay run for 16 hours; data given are means of triplicates ± SE of mean. Since the culture with BALB/c-H-2<sup>db</sup> stimulators gave a higher yield and more powerful cytotoxicity, data presented are from effector:target ratios where lysis caused by both T cell populations is similar.

- a Cytotoxic T cells were generated in secondary responses *in vitro* to syngeneic ectromelia-infected cells (see Materials and Methods).
- b H-2 map regions as for Table 1.
- c Blocking antiserum was added to targets before addition of T cells. Full procedure given in Materials and Methods. Nil = no serum.
- d Assay run for 4 hours at an effector:target ratio of 2:1. Data given are means of triplicates  $\pm$  SE of mean with spontaneous release subtracted. Lysis of uninfected controls was negligible.
- e Significantly less lysis than with anti-H-2.4 and anti-H-2.28 sera ( $P < 0.05$ ).
- f Significantly less lysis than with anti-H-2.31 serum ( $P < 0.05$ ).
- g Significantly less lysis than with anti-H-2.31 and anti-H-2.28 sera ( $P < 0.01$ ).



TABLE 6

BLOCKING OF T-CELL-MEDIATED LYSIS OF VIRUS-INFECTED MACROPHAGES BY  
"MONOSPECIFIC" ANTI-H-2 SERA

Donors of virus-specific T cells <sup>a</sup>	H-2 region shared with target	Specificity of blocking anti-serum <sup>c</sup>	% Specific lysis of infected targets <sup>d</sup>	
			BALB/cKh	BALB/c-H-2 <sup>db</sup>
D2.GD <sup>b</sup> (ddbbbb)	K <sup>d</sup>	H-2.4 (D <sup>d</sup> )	25.9 ± 2.0	41.9 ± 1.5
		H-2.28 (D <sup>d</sup> )	25.1 ± 2.4	40.7 ± 1.8
		H-2.31 (K <sup>d</sup> )	19.4 ± 1.7 <sup>e</sup>	32.7 ± 2.6 <sup>e</sup>
		Nil	35.2 ± 0.6	43.8 ± 2.2
A.TH (sssssd)	D <sup>d</sup>	H-2.4 (D <sup>d</sup> )	7.3 ± 2.4 <sup>f</sup>	8.2 ± 1.5 <sup>g</sup>
		H-2.28 (D <sup>d</sup> )	9.6 ± 1.5 <sup>f</sup>	19.1 ± 1.7
		H-2.31 (K <sup>d</sup> )	18.0 ± 2.9	20.2 ± 0.9
		Nil	17.4 ± 0.9	16.9 ± 1.6

Cytotoxic T cells (T<sub>C</sub> cells) generated in response to virus-infected or hapten-modified self cells exhibit H-2 restriction, i.e., they apparently recognize antigenic patterns presented on self H-2B or H-2D antigens plus the foreign antigen (Doherty et al., 1976; Shewar et al., 1976). Models to explain this phenomenon fall into two general categories. The first (dual-recognition) states that an individual T<sub>C</sub> cell expresses on its surface membrane two different types of antigen binding sites, one specific for a self H-2B or H-2D antigenic determinant, and the other specific for a foreign antigenic determinant (1). There are many points of detail which distinguish these two models (Doherty et al., 1976; Shewar et al., 1976; Langman, 1977; Bland and Shewar, 1977), but they all depend on the pairing of binding sites (anti-H-2 plus anti-foreign antigen) in triggering T<sub>C</sub> cell function, whereas binding to self H-2 sites, or to self H-2B or H-2D sites, is not considered to be essential for T<sub>C</sub> cell function.

#### SPECIFIC ADSORPTION OF H-2-RESTRICTED

#### CYTOTOXIC T CELLS TO MACROPHAGE

#### MONOLAYERS

The binding site for an antigenic determinant is a complex of self H-2B or H-2D plus a foreign antigenic determinant. This complex can be viewed as a "new antigenic determinant" formed by the change in one or the other, or both, of the interacting antigenic determinants, but nonetheless dependent for its specificity on both components. Alternatively, it can be viewed as a "junction zone" antigenic determinant formed by a complex of the self H-2 and foreign molecules and containing a component from both of them. In this latter case, however, the T<sub>C</sub> cell binding site must have affinity only for the complex and not for each of the separate components or it becomes, by definition, a case of dual recognition.



## INTRODUCTION

Cytotoxic T cells ( $T_C$  cells) generated in response to virus-infected or hapten-modified self cells exhibit H-2 restriction, i.e., they apparently recognize antigenic patterns dependent on self H-2K or H-2D antigens plus the foreign antigen (Doherty *et al.*, 1976a; Shearer *et al.*, 1976). Models to explain this phenomenon fall into two general categories. The first (dual recognition) states that an individual  $T_C$  cell expresses on its surface membrane two different types of antigen binding sites, one specific for a self H-2K or H-2D antigenic determinant, and the other specific for a foreign antigenic determinant (X). There are many points of detail which distinguish different versions of this model (Doherty *et al.*, 1976b; Janeway *et al.*, 1976; Blanden *et al.*, 1977; Langman, 1977; Blanden and Ada, 1978), but they all demand a paired set of binding sites (anti-H-2 plus anti-X) to be essential in triggering  $T_C$  cell function, whereas antigen binding to two anti-H-2 sites, or two anti-X sites is not operationally important. The second model (altered self) states that an individual  $T_C$  cell expresses only one type of antigen binding site which is specific for an antigenic pattern that is dependent upon an interaction or complex between a self H-2K or H-2D molecule and a foreign antigen. This antigenic pattern can be viewed as a "new antigenic determinant" caused by allosteric change in one or the other, or both, of the interacting antigenic components, but nonetheless dependent for its specificity on both components. Alternatively, it can be viewed as a "junction zone" antigenic determinant formed by a complex of the self H-2 and foreign molecules and containing a component from both of them. In this latter case however, the  $T_C$  cell binding site must have affinity only for the complex and not for each of the separate components or it becomes, by definition, a class of dual recognition.

Thus far, there is no conclusive evidence which excludes either model. However, the exquisite specificity of self H-2 recognition, as illustrated particularly by *H-2K* mutants (Blanden *et al.*, 1976; Kees and Blanden, 1976; Zinkernagel, 1976; McKenzie *et al.*, 1977), the wide variety of viral and other antigens which cause H-2 restricted responses (Doherty *et al.*, 1976a; Shearer *et al.*, 1976; Blanden *et al.*, 1977) and considerations of  $T_C$  cell ontogeny (Langman, 1977; Zinkernagel *et al.*, 1978; Blanden and Ada, 1978) impose severe strain on altered self models. On the other hand, cold target competition experiments are readily explained by altered self, but require qualification for dual recognition. The salient observations are as follows:  $T_C$  cell-mediated lysis of radio-labelled virus-infected target cells is specifically inhibited only by addition of virus-infected unlabelled competitors which have the same H-2 antigens as the labelled targets; H-2 compatible uninfected competitors, or infected allogeneic competitors are not specifically inhibitory. The precise mechanism of competition is unknown, but several possibilities may be considered. First, it may be simply a case of competition for  $T_C$  cell antigen-binding sites. If this be the case, the problem for dual recognition is that multipoint binding of  $T_C$  cells to either H-2 or X antigens, not necessarily both, should cause competition. Dual recognition can be salvaged, however, by postulating that cold competition operates through the inability of  $T_C$  cells to lyse more than one target cell simultaneously (Bevan *et al.*, 1976; Langman, 1977). Thus, it could be that while many targets bind simultaneously to an individual  $T_C$  cell, only one is being lysed within a certain finite time period.

The present experiments address this problem by testing whether  $T_C$  cells specific for viral antigen plus self H-2 bind to macrophage monolayers displaying either viral antigens with allogeneic H-2, self H-2



but no viral antigen, or both viral and self H-2 antigens together. Specific binding could be demonstrated only in the last case, a result compatible with altered self and requiring further operational assumptions in dual recognition models.

## MATERIALS AND METHODS

### Mice

Mice were bred at the John Curtin School and were used at 6-8 weeks of age. In any one experiment, mice were of the same sex.

### Viruses

Ectromelia virus stocks of both virulent (Moscow) and attenuated (Hampstead egg) strains were used. The methods of growing and titrating these strains have been described in Chapter 2. Sendai and influenza virus strain A/JAP were grown and titrated as described by Doherty and Zinkernagel (1976) and Yap and Ada (1977) respectively.

### Immunization

Mice were infected i.v. with attenuated ectromelia virus (Gardner *et al.*, 1974), influenza virus strain A/JAP (Yap and Ada, 1977) and Sendai virus (Doherty and Zinkernagel, 1976) as described previously. Spleen cells were used as a source of memory responder cells 3-5 weeks later.

### Preparation of TNP (trinitrophenyl)-conjugated stimulator and target cells

The method used was basically similar to that reported by other workers (Shearer *et al.*, 1975; Forman, 1975). Briefly, normal splenic lymphocytes or L929 fibroblasts were modified with 10mM TNBS (trinitrobenzenesulphonic acid) in phosphate-buffered saline (PBS) for 10 min at 37°C and thoroughly washed with PBS prior to and post-modification.

### Generation of effector T<sub>C</sub> cells *in vitro*

The method used was described for ectromelia virus in Chapter 3.

Briefly, spleen cells from mice previously immunized with virus (ectromelia, influenza or Sendai) were cultured with syngeneic splenic stimulator cells infected with the same virus as used for immunization. 2 PFU virulent ectromelia virus/cell was used for the infection of stimulator cells. The other stimulator cells were infected with 1-2 EID<sub>50</sub> units of Sendai virus or 2 EID<sub>50</sub> units of influenza virus strain A/JAP. A stimulator:responder ratio of 1:10 was used for all viral systems. Cultures were set up in tissue culture flasks (Falcon Plastics, 75 cm<sup>2</sup> growth area) at a final concentration of  $2 \times 10^6$  responder cells/ml in 40 ml/flask. Usually  $8 \times 10^7$  responder cells were cultured with  $8 \times 10^6$  stimulator cells at 37°C in an atmosphere of 10% CO<sub>2</sub> for 5 days. (Cultures using ectromelia virus were incubated at 39°C, a non-permissive temperature for ectromelia replication, so preventing the virus from exerting cytopathic effects against responder cells.) The complete culture medium was Eagle's minimal essential medium (GIBCO cat. no. F-15) with non-essential amino acids, 10% foetal calf serum,  $10^{-4}$  M 2-mercaptoethanol and antibiotics.

The generation *in vitro* of secondary TNP-immune T<sub>C</sub> cells was essentially as for virus-immune T<sub>C</sub> cells. TNP-modified spleen cells ( $2 \times 10^7$  per culture) were used as stimulators with  $8 \times 10^7$  responder cells from mice primed 2-12 weeks previously with TNP-modified syngeneic spleen cells. Immunization with TNP-modified spleen cells was obtained by subcutaneous injection of  $4 \times 10^6$  TNP-modified spleen cells in 40 µl PBS into the hind footpad.

For primary one-way mixed lymphocyte reactions (MLR) splenic responder cells and γ-irradiated (1000 Rads), allogeneic splenic stimulator cells were cultured at 37°C for 5 days at a responder:stimulator ratio of 4:1 (Gardner *et al.*, 1975).



### Removal of dead cells

After each harvest of responder cells from cultures, dead cells were removed by centrifuging through Ficoll-Isopaque as described in Chapter 3.

### Adsorption of immune lymphocytes on macrophage monolayers

The method described by Brondz *et al.* (1975) was modified as follows: Peritoneal macrophages were harvested 3 days after intraperitoneal injection of an irritant (4 ml Thioglycollate medium, DIFCO, Detroit, Michigan, USA). Almost confluent monolayers were prepared by seeding  $7 \times 10^6$  peritoneal macrophages in 10 ml F-15 containing 10% FCS in a tissue culture flask ( $25 \text{ cm}^2$  growth area, Falcon Plastics) and incubating overnight at  $37^\circ\text{C}$ . Monolayers were rigorously washed with warm medium to remove all non-adherent cells; the cells remaining in the flask were then uniformly large, strongly adherent macrophages. They were then either left untreated or were infected with virus or modified with TNBS as described for target cells used in the cytotoxicity assay. Following washing, all medium was drained from the flasks and 2 ml of complete medium containing  $10^7$  lymphoid cells (including effector  $T_C$  cells) from the cultures described above were added to each flask. Adsorption was achieved by incubating at  $30^\circ\text{C}$  for 2.5 hrs in a horizontal position. The non-adherent cells were collected by removing the supernatant with a pipette without prior agitation. This procedure inevitably resulted in some loss of non-adherent cells in the small volume of medium remaining in the flask. There could also be a degree of nonspecific adherence and trapping as reported in several laboratories (Brondz *et al.*, 1975; Neefe and Sachs, 1976; Geib *et al.*, 1978). However, this method avoided the problems of detachment of cells from the adsorbing monolayer which would then contaminate the effector cell suspension and act as cold competitors

(Neeffe and Sachs, 1977). Because of their large size and morphology, Thioglycollate-induced macrophages which became detached could be easily identified during counting of the non-adherent cells harvested after the adsorption procedure. They were never more than 1.5% of the total non-adherent cell population, which was then tested for effector activity on  $^{51}\text{Cr}$  labelled target cells. Since the maximum effector:target ratio used was 10:1, this level of contamination was never high enough for the macrophages to act as cold competitors.

#### Cytotoxicity assay

The methods used for L929 and macrophage targets have been described in detail in Chapter 3. P815 cells were treated as L929 cells and were grown in Eagle's minimal essential medium (GIBCO, cat. no. H-16) containing 10% FCS. Target cells were infected with 2 EID<sub>50</sub> of Sendai or influenza virus strain A/JAP. Data given are the means of triplicates and have had spontaneous release subtracted for assay times of 6 hrs at 37°C. Standard errors of the means were always < 3% and are omitted for clarity. Significance was determined by Student's t test.

#### RESULTS

##### Specific depletion of $T_c$ cells generated in MLR

The specificity and efficiency of the monolayer adsorption procedure was first established for alloreactive  $T_c$  cells. CBA/H (H-2<sup>k</sup>) anti-A.TL (H-2K<sup>S</sup>, H-2D<sup>d</sup>) effector cells were tested on SJL/J (H-2<sup>S</sup>) and BALB/c (H-2<sup>d</sup>) macrophage targets after adsorption on either SJL/J or BALB/c macrophage monolayers. The results (Figure 1) indicate that  $T_c$  cell clones reactive to H-2K<sup>S</sup> bound significantly more to SJL/J than to BALB/c adsorbing monolayers (left panel). The converse applied to  $T_c$  cell clones reactive to H-2D<sup>d</sup> (right panel). There was a



degree of nonspecific loss of effector activity inherent in the adsorption procedure for reasons given in Materials and Methods. The degree of reduction of anti-H-2K<sup>S</sup> activity caused by adsorption on BALB/c monolayers in Figure 1 was similar to that caused by adsorption on monolayers syngeneic with the responding T<sub>C</sub> cells, or on allogeneic monolayers unrelated to either responders or stimulators (data not shown). In the remaining experiments, appropriate control monolayers serve as a basis for determining specific adsorption.

#### Specific depletion of T cells sensitized to TNP-modified or virus-infected cells

CBA/H effector cells generated against TNP-modified or influenza virus-infected syngeneic spleen cells were placed on a panel of monolayers of CBA/H or BALB/c macrophages, either TNP-modified, infected with influenza virus (JAP) or untreated (Table 1). Specific adsorption occurred only when monolayers were homologous with the stimulator cells used in culture, i.e., CBA/H TNP-immune T<sub>C</sub> cells were specifically adsorbed only on TNP-modified CBA/H monolayers. Further, CBA/H influenza-immune T<sub>C</sub> cells adsorbed specifically on influenza virus-infected CBA/H monolayers while no reduction of cytotoxicity was observed after contact with TNP-modified or virus-infected BALB/c monolayers. To ensure that the BALB/c macrophages were capable of specific adsorption, alloreactive T<sub>C</sub> cells generated in a MLR were tested on the same panel of monolayers. Adsorption of anti-BALB/c T<sub>C</sub> cells occurred on all BALB/c monolayers irrespective of infection by influenza virus or TNP modification.

These observations were extended to include BALB/c T<sub>C</sub> cells. Influenza-immune T<sub>C</sub> cells from BALB/c mice can be specifically adsorbed on the appropriate virus-infected monolayers (Table 2).

#### Virus-specificity of adsorption

To test the specificity of adsorption of virus-immune T<sub>C</sub> cells,

Sendai and influenza viruses were used. Table 3 illustrates that adsorption is highly virus-specific. Both types of virus-immune  $T_C$  cells showed significantly reduced cytotoxicity only after contact with syngeneic monolayers infected with the same virus as used for immunization.

#### DISCUSSION

The present experiments showed that  $T_C$  cell subsets which lyse allogeneic or various virus-infected or TNP-modified self or H-2 compatible targets can be specifically depleted from cell suspensions by adsorption onto appropriate Thioglycollate-stimulated macrophage monolayers. Adsorption of alloreactive  $T_C$  cells has been reported previously by several groups (Kedar *et al.*, 1974; Brondz *et al.*, 1975; Neefe and Sachs, 1976) using various types of adsorbing cell layers. In a single adsorption cycle, the method used here, it consistently gave 3-fold specific depletion of effector activity below the small amount of nonspecific depletion inherent in the procedure. Three-fold specific depletion was ample for the main purpose of this report, though a second cycle of adsorption can be used to reduce effector activity by 10-fold or more (unpublished data).

Our prime objective was to determine whether  $T_C$  cells which recognize antigenic patterns dependent on both self H-2 and a given foreign antigen (X) could bind specifically to macrophages displaying (a) self H-2 alone; (b) X alone, or (c) self H-2 plus X together. The results clearly indicated that significant specific binding occurred only in case (c) i.e., where the adsorbing macrophages displayed both self H-2 and the X antigen used for immunization. This adsorption was specific for both H-2 and X antigens in the examples tested, i.e., there was clear discrimination between  $H-2^k$  and  $H-2^d$  (acting as "self markers"), and between TNP,



influenza (JAP) and Sendai viral antigens (acting as X). Since no specific adsorption of H-2 restricted  $T_c$  cells (specific for self H-2 plus X) could be detected on either H-2-bearing or on X-bearing macrophages, these results are consistent with "altered self" as defined in the Introduction. At face value they are not compatible with dual recognition models in which multiple copies of anti-H-2 and anti-X binding sites, each with measurable affinity for antigen, are displayed on  $T_c$  cell surface membranes and are thus available to participate in multipoint (high avidity) interaction with either self H-2 or with X antigenic determinants on adsorbing cell surface membranes.

However, the biophysical and genetic problems inherent in altered self models in the light of available information (see refs. Langman (1977); Blanden and Ada (1978) for review of salient points) may justify formulation and testing of modifications of dual recognition models which can accommodate the present data. Such modifications necessarily invoke new assumptions concerning the expression and control of  $T_c$  cell receptors and/or the mechanisms of antigen-dependent triggering of lytic function as a consequence of receptor-antigen interaction.

#### SUMMARY

Adsorption on macrophage monolayers was used to determine the binding specificity of cytotoxic T cells generated in response to virus-infected or hapten-modified self-cells. The results clearly indicate that significant specific binding occurred only in cases where the adsorbing macrophages displayed both viral (or hapten) and self H-2 antigens together. There was clear discrimination between  $H-2^k$  and  $H-2^d$  (acting as "self markers"), and between TNP, influenza (JAP) and Sendai viral antigens (acting as foreign antigen). These results are consistent with an "altered self" model as an explanation for H-2 restriction.

## REFERENCES

- Bevan, M. J., Langman, R. E. and Cohn, M. 1976. H-2 antigen-specific cytotoxic T cells induced by concanavalin A: estimation of their relative frequency. *Eur.J.Immunol.* 6:150.
- Blanden, R. V., Dunlop, M. B. C., Doherty, P. C. Kohn, H. I. and McKenzie, I. F. C. 1976. Effects of four H-2K mutations on virus-induced antigens recognized by cytotoxic T cells. *Immunogenetics* 3:541.
- Blanden, R. V., Pang, T. E. and Dunlop, M. B. C. 1977. T cell recognition of virus-infected cells, in: *Virus infection and the cell surface, Cell Surface Reviews, Vol. 2*, G. Poste and G. L. Nicolson, eds., North Holland Publishing Company, Amsterdam, p. 249.
- Blanden, R. V. and Ada, G. L. 1978. A dual recognition model for cytotoxic T cells based on thymic selection of precursors with low affinity for self H-2 antigens. *Scand.J.Immunol.* in press.
- Brondz, B. D., Egorov, I. K. and Drizlikh, G. I. 1975. Private specificities of H-2K and H-2D loci as possible selective targets for effector lymphocytes in cell-mediated immunity. *J.Exp.Med.* 141:11.
- Doherty, P. C. and Zinkernagel, R. M. 1976. Specific immune lysis of paramyxovirus-infected cells by H-2 compatible thymus-derived lymphocytes. *Immunology* 31:27.
- Doherty, P. C., Blanden, R. V. and Zinkernagel, R. M. 1976a. Specificity of virus-immune effector T cells for H-2K or H-2D compatible interactions: implications for H-antigen diversity. *Transplant. Rev.* 29:89.
- Doherty, P. C., Goetze, D., Trinchieri, G. and Zinkernagel, R. M. 1976b. Models for recognition of virally modified cells by immune thymus-derived lymphocytes. *Immunogenetics* 3:517.
- Forman, J. 1975. On the role of the H-2 histocompatibility complex in determining the specificity of cytotoxic effector cells sensitized against syngeneic trinitrophenyl-modified targets. *J.Exp.Med.* 142:403.
- Gardner, I. D., Bower, N. A. and Blanden, R. V. 1974. Cell-mediated cytotoxicity against ectromelia virus-infected targets. I. Specificity and kinetics. *Eur.J.Immunol.* 4:63.
- Gardner, I. D., Bower, N. A. and Blanden, R. V. 1975. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. III. Role of the H-2 gene complex. *Eur.J.Immunol.* 5:122.
- Geib, R., Chiang, C. and Klein, J. 1978. Evidence for multiple clones of cytotoxic T cells responding to antigenic determinants on the same molecule. *J.Immunol.* 120:340.



- Janeway Jr, C. A., Wigzell, H. and Binz, H. 1976. Two different V<sub>H</sub> gene products make up the T cell receptors. *Scand.J.Immunol.* 5:993.
- Kedar, E., Ortiz de Landazuri, M., Bonavida, B. and Fahey, J. L. 1974. Cellular immunoadsorbents. An improved technique for specific depletion of cytotoxic (T) lymphoid cells. *J.Immunol. Methods* 5:97.
- Kees, U. and Blanden, R. V. 1976. A single genetic element in H-2K affects mouse T-cell antiviral function in poxvirus infection. *J.Exp.Med.* 143:450.
- Langman, R. E. 1977. Cell-mediated immunity and the major histocompatibility complex. *Rev. Phys. Biochem. Pharm.* 81:1.
- McKenzie, I. F. C., Pang, T. and Blanden, R. V. 1977. The use of H-2 mutants as models for the study of the T cell activation. *Immunol. Rev.* 35:181.
- Neefe, J. R. and Sachs, D. H. 1976. Specific elimination of cytotoxic effector cells. I. Adsorptive behaviour of effectors and their precursors on spleen cell monolayers. *J.Exp.Med.* 144:996.
- Neefe, J. R. and Sachs, D. H. 1977. Specific elimination of cytotoxic cells. II. Reduction of cytotoxic activity by adsorption on monolayers results from removal of cytotoxic cells and not from inhibition of their activity. *Cell.Immunol.* 29:129.
- Shearer, G. M., Rehn, T. G. and Garbarino, C. A. 1975. Cell mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the H-2K and H-2D serological regions of the murine major histocompatibility complex. *J.Exp.Med.* 141:1348.
- Shearer, G. M., Rehn, T. G. and Schmitt-Verhulst, A. 1976. Role of the murine major histocompatibility complex in the specificity of *in vitro* T cell-mediated lympholysis against chemically modified autologous lymphocytes. *Transplant. Rev.* 29:222.
- Yap, K. L. and Ada, G. L. 1977. Cytotoxic T cells specific for influenza virus-infected target cells. *Immunology* 32:151.
- Zinkernagel, R. M. 1976. H-2 compatibility requirement for virus-specific T cell mediated cytolysis. The H-2K structure is coded by a single cistron defined by H-2K<sup>b</sup> mutant mice. *J.Exp.Med.* 143:437.
- Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Klein, P. A. and Klein, J. 1978. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J.Exp.Med.* 147:882.

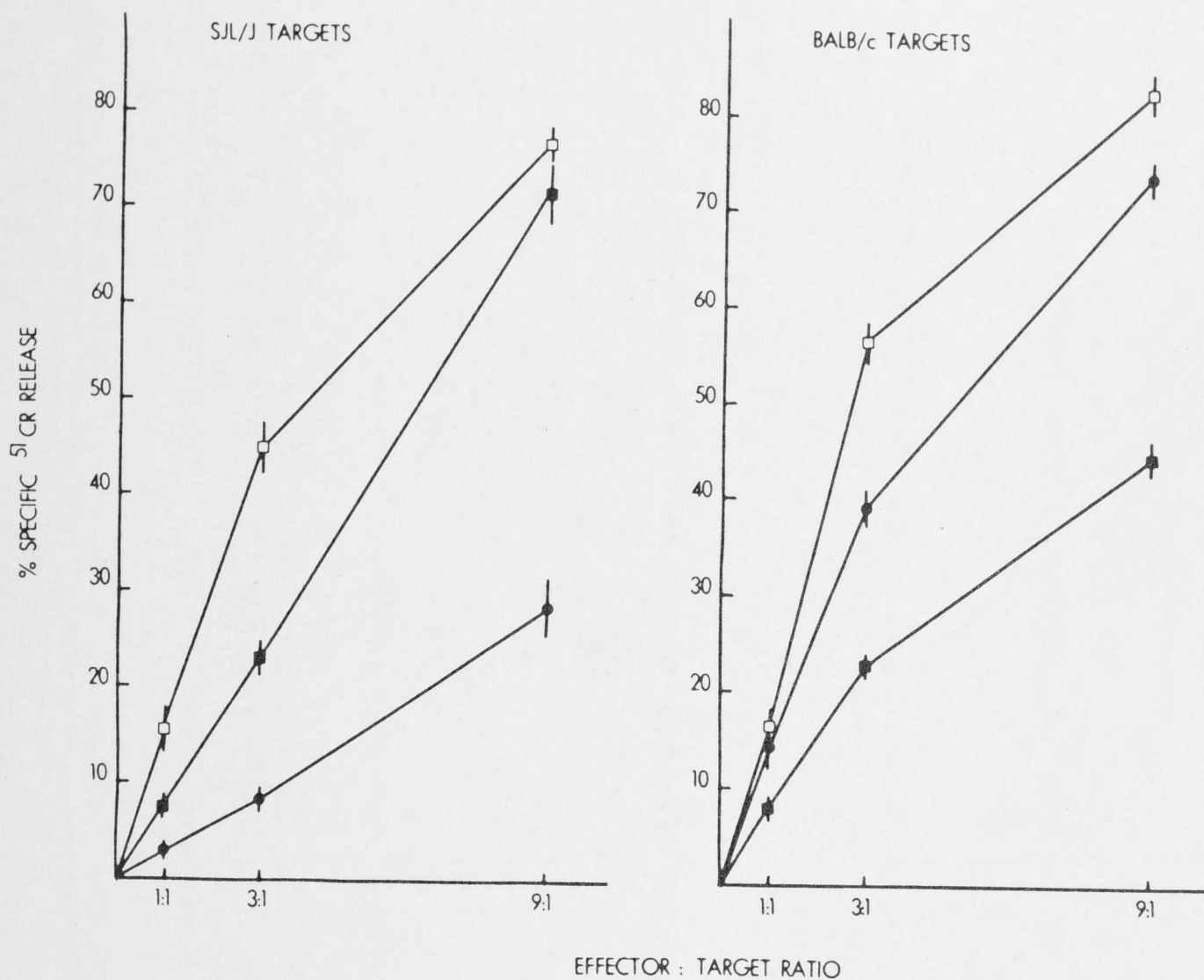


FIG. 1 - Specific cytotoxic activity of MLR cells from CBA/H ( $H-2^k$ ) cells generated against A.TL ( $H-2K^S$ ,  $H-2D^d$ ) cells tested at indicated effector target ratio on macrophage targets from SJL/J( $H-2^S$ ) (left panel) or BALB/c ( $H-2^d$ ) mice (right panel).  $T_C$  cells were adsorbed on SJL/J ( $H-2^S$ ) monolayers (●—●), on BALB/c ( $H-2^d$ ) monolayers (■—■) or unadsorbed (□—□). Vertical bars enclose 2 s.e. of the mean.



- a Data given are means of triplicates of % specific  $^{51}\text{Cr}$  release at effector:target ratios of 3:1 and 10:1.
- b Effector  $T_c$  cells were generated *in vitro* in secondary responses in the case of TNP and influenza virus (JAP) or in primary MLR.
- c Significantly less than appropriate controls at the same effector:target ratio ( $P < 0.001$ ).

TABLE 1

SPECIFIC ADSORPTION OF TNP-IMMUNE, VIRUS-IMMUNE AND  
ALLOREACTIVE T<sub>c</sub> CELLS ON MACROPHAGE MONOLAYERS<sup>a</sup>

Adsorption monolayers	CBA/H anti-CBA/H-TNP T <sub>c</sub> cells <sup>b</sup> on TNP-L929(H-2 <sup>k</sup> ) targets		CBA/H anti-CBA/H-JAP T <sub>c</sub> cells <sup>b</sup> on JAP-L929(H-2 <sup>k</sup> ) targets		CBA/H anti-BALB/c T <sub>c</sub> cells <sup>b</sup> on P815(H-2 <sup>d</sup> ) targets	
	3:1	10:1	3:1	10:1	3:1	10:1
CBA/H (H-2 <sup>k</sup> )	46.1	70.6	26.1	49.5	29.0	71.1
CBA/H-TNP	12.0 <sup>c</sup>	30.1 <sup>c</sup>	34.8	59.6	39.3	79.9
CBA/H-JAP	49.8	88.0	12.8 <sup>c</sup>	29.2 <sup>c</sup>	44.0	79.6
BALB/c(H-2 <sup>d</sup> )	53.0	83.9	35.0	59.2	8.9 <sup>c</sup>	29.6 <sup>c</sup>
BALB/c-TNP	42.2	81.3	28.5	50.5	9.4 <sup>c</sup>	27.6 <sup>c</sup>
BALB/c-JAP	44.7	85.4	26.7	45.7	2.5 <sup>c</sup>	15.5 <sup>c</sup>



TABLE 2

SPECIFIC ADSORPTION OF BALB/c INFLUENZA (JAP)-IMMUNE T<sub>c</sub> CELLS<sup>a</sup>

Adsorption monolayers	Effector:target ratio	% Specific <sup>51</sup> Cr release from P815 (H-2 <sup>d</sup> ) targets <sup>b</sup>	
		JAP-infected	uninfected
CBA/H (H-2 <sup>k</sup> )	3:1	49.9	1.5
	10:1	77.1	0.1
BALB/c (H-2 <sup>d</sup> )	3:1	50.5	0.5
	10:1	83.4	1.0
BALB/c-JAP	3:1	11.3 <sup>c</sup>	1.1
	10:1	52.7 <sup>c</sup>	2.0

a Effector cells were generated in secondary responses *in vitro*.

b Data given are means of triplicates.

c Significantly lower ( $P < 0.001$ ) than all other groups on infected target cells tested at the same effector:target ratio.

TABLE 3

VIRUS-SPECIFIC ADSORPTION OF CBA/H T<sub>C</sub> CELLS STIMULATED BY SENDAI OR INFLUENZA (JAP) VIRUSES<sup>a</sup>

Adsorption monolayer	Sendai-immune T <sub>C</sub> cells <sup>b</sup> on Sendai-L929 (H-2 <sup>k</sup> ) targets		JAP-immune T <sub>C</sub> cells <sup>b</sup> on JAP-L929 (H-2 <sup>k</sup> ) targets	
	3:1	10:1	3:1	10:1
CBA/H (H-2 <sup>k</sup> )	15.9	38.2	26.5	59.9
CBA/H-JAP	17.3	36.6	15.7 <sup>c</sup>	36.8 <sup>c</sup>
CBA/H-Sendai	7.8 <sup>c</sup>	14.7 <sup>c</sup>	24.1	54.9
BALB/c (H-2 <sup>d</sup> )	18.3	38.4	32.5	60.4
BALB/c-Sendai	14.7	31.1	35.4	60.2

a Data given are means of triplicates of % specific <sup>51</sup>Cr release at effector:target ratios of 3:1 and 10:1.

b Effector T<sub>C</sub> cells were generated *in vitro* in secondary responses.

c Significantly less lysis than all other groups on the same target at the same effector:target ratio (P < 0.001).



T cell mediated lysis of ectromelia virus-infected target cells requires H-2K or H-2D region sharing between donors of cytotoxic T cells and infected targets. The same restriction holds for effector cells generated in primary and secondary responses *in vitro* (Chapter 4) and for the antiviral effect of spleen cells from immune donors (Chapter 5). In fact, the donors of immune cells have to share either H-2K or H-2D region genes with the recipients for transfer of virus clearance mechanisms with spleen cells to occur. Available evidence suggests that this reflects a restricted repertoire of recognition structures in the cytotoxic T cell pool.

Cytotoxic T(?) cells responding to viral infection see self H-2 in connection with viral antigen; not viral antigen alone. The question arises whether this reflects "syngeneic preference", i.e., are T cells committed to recognise self-H-2 but not non-self-H-2 together with viral antigen?

Several attempts have been made to determine whether all genetic factors determining compatibility control the cytotoxic response to allogeneic cells.

#### CYTOTOXIC T CELL RESPONSE TO ALLOGENEIC

##### ECTROMELIA VIRUS-INFECTED CELLS

influence over induction of cytotoxicity. The main problem with this approach is that much of the cytotoxic activity generated against such stimulator cells is directed towards allogeneic cells per se. To avoid this problem, several methods of generating responder cells responsive to the alloantigen in question have been devised. We used radiation chimeras (von Boehmer et al., 1975) and neonatally tolerant mice (Billingham et al., 1953). Lethally irradiated F<sub>1</sub> hybrid mice were repopulated with equal proportions of foetal liver cells from both parental strains (Gallwey et al., 1975). This procedure allows stem cell differentiation in the absence of mature T cells in a histoincompatible environment, a situation possibly resembling that of physiological stem cell differentiation during T cell

## INTRODUCTION

T cell mediated lysis of ectromelia virus-infected target cells requires *H-2K* or *H-2D* region sharing between donors of cytotoxic T cells and infected targets. The same restriction holds for effector cells generated in primary and secondary responses *in vitro* (Chapter 4) and for the antiviral effect of spleen cells from immune donors (Chapter 2), i.e., the donors of immune cells have to share either *H-2K* or *H-2D* region genes with the recipients for transfer of virus clearance mechanisms with spleen cells to occur. Available evidence suggests that this reflects a restricted repertoire of recognition structures in the cytotoxic T cell pool. Cytotoxic T( $T_c$ ) cells responding to viral infection see self H-2 in connection with viral antigen, not viral antigen alone. The question arises whether this reflects "syngeneic preference", i.e., are  $T_c$  cells committed to recognize self-H-2 but not non-self-H-2 together with viral antigens? Several attempts have been made to determine whether allogeneic major histocompatibility complex (MHC) gene products exert a similar restrictive influence over induction and effector function of cytotoxic T cells generated against virus-infected allogeneic cells. The main problem with this approach is that much of the cytolytic activity generated against such stimulator cells is directed towards allogeneic cells *per se*. To avoid this problem, several methods of rendering responder cells unresponsive to the alloantigen in question have been devised. We used radiation chimeras (von Boehmer *et al.*, 1975) and neonatally tolerant mice (Billingham *et al.*, 1953). Lethally irradiated  $F_1$  hybrid mice were repopulated with equal proportions of foetal liver cells from both parental strains (Tulunay *et al.*, 1975). This procedure allows stem cell differentiation in the absence of mature T cells in a histoincompatible environment, a situation possibly resembling that of physiological stem cell differentiation during T cell



ontogeny in normal mice. Chimeric cells of the two lymphocyte populations appear to be mutually and specifically tolerant (von Boehmer *et al.*, 1975). We report here that  $T_C$  cells from such chimeras can lyse allogeneic ectromelia virus-infected target cells. Recent experiments with grafted thymuses have demonstrated that an essential host influence comes from a radioresistant thymic component, presumably epithelium (Zinkernagel *et al.*, 1977). If  $(P_1 \times P_2) F_1$  stem cells are allowed to differentiate into mature  $T_C$  cells in irradiated  $P_1$  or  $P_2$  host mice, only one of the two potential  $F_1 T_C$  cell subsets can be detected in H-2 restricted responses to minor H (Bevan, 1977) or viral antigens (Zinkernagel *et al.*, 1978a): only H-2 antigens present in the irradiated host seems to be recognized. Two concepts to visualize the selection process applied to progenitor  $T_C$  cells in the thymus have been proposed: A "positive selection" model (Langman, 1977; Zinkernagel *et al.*, 1978a) or a "negative selection" model (Blanden and Ada, 1978). In this report we present indirect evidence consistent with a negative selection model.

## MATERIALS AND METHODS

### Mice

Inbred mice were bred at the John Curtin School and were used at 6 - 8 weeks of age. In any one experiment mice of the same sex were used.

### Chimeras

$F_1$  (CBA/H  $\times$  C57BL/6) ( $H-2^k \times H-2^b$ ) mice were exposed to 850 to 950 Rads of total body irradiation from a  $^{60}\text{Co}$  source and were injected i.v. within 24 hours with  $2 \times 10^7$  foetal liver cells from 16- to 19-day-old foetuses ( $10^7$  CBA/H and  $10^7$  C57BL/6 cells). These mice are referred to as  $(P_1 + P_2) \rightarrow F_1$  chimeras. Chimeras of the type  $F_1 \rightarrow P_1$  or  $F_1 \rightarrow P_2$  (e.g., CBA/H mice reconstituted with  $2 \times 10^7$  (CBA/H  $\times$  C57BL/6) $F_1$  foetal liver cells) were prepared similarly. Mice were given antibiotics in their

drinking water for three weeks after irradiation and were used in experiments 6 - 10 weeks after reconstitution.

#### Preparation of neonatally tolerant mice

Tolerance was induced as described by Brooks (1975). Within 24 hours of birth CBA/H (H-2<sup>k</sup>) or C57BL/6 (H-2<sup>b</sup>) mice were inoculated intraperitoneally with  $5 \times 10^7$  (CBA/H x C57BL/6) F<sub>1</sub> spleen cells in a volume of 0.1 ml. Only mice that had been injected satisfactorily technically were returned to their mothers. Spleen cells from mice injected at birth were used in experiments at 6 - 8 weeks of age. The assessment of tolerance was an integral part of experiments carried out *in vitro*. Spleen cells from these mice were used as responder cells with ectromelia virus-infected (CBA/H x C57BL/6) F<sub>1</sub> spleen cell stimulators, cultured for 5 days at 39°C as indicated below and tested for lysis of infected and uninfected target cells of both parental haplotypes. Absence of lysis of uninfected target cells of the haplotype to which tolerance was induced indicated that all nine CBA/H mice were indeed tolerant to C57BL/6, whereas only one out of nine C57BL/6 mice appeared to be tolerant to CBA/H. It has been known for some time that transplantation tolerance in C57BL mice across the H-2 barrier is extremely difficult to achieve (Billingham and Brent, 1957). Streilein and Klein (1977) reported that genetic factors unlinked to the H-2 complex seem to play a role in determining the ease of neonatal tolerance induction.

#### Viruses

Stocks of attenuated (Hampstead egg strain) and virulent (Moscow strain) ectromelia virus were prepared, titrated and stored as described in Chapter 2.

#### Immunization

Mice were immunized i.v. with  $2 \times 10^4$  PFU of attenuated ectromelia



virus and their spleens were harvested 5 days later.

#### Estimation of lymphoid cell chimerism

Antisera: Anti-CBA/H (anti-H-2<sup>k</sup>) and anti-C57BL/6 (anti-H-2<sup>b</sup>) sera were produced by injecting C57BL/6 or CBA/H mice at weekly intervals with  $2 \times 10^7$  CBA/H or C57BL/6 spleen cells respectively i.v. for seven weeks. The animals were bled at the end of the 8th week. The sera were absorbed with syngeneic spleen cells (1 ml serum was mixed with  $3 \times 10^8$  spleen cells and incubated for 30 minutes at 0°C) and stored at -70°C.

Cytotoxicity assay: Spleen cells freed of dead cells (see below) were suspended at a concentration of  $4 \times 10^6$  cells/ml in F-15 containing anti-serum. Antisera dilutions were chosen which gave > 95% lysis of (CBA/H x C57BL/6) F<sub>1</sub> spleen cells. The cells were incubated for 30 minutes at room temperature, washed twice and then incubated with (1:4) diluted rabbit complement (in F-15) for 45 minutes at 37°C at a concentration of  $8 \times 10^6$  cells/ml. (Any cytotoxicity of the rabbit serum was removed by repeated absorption with spleen cell suspensions of CBA/H and C57BL/6 mice under conditions as given for the absorption of the antisera). The proportion of live cells after treatment was estimated by Trypan Blue exclusion.

#### Primary *in vitro* responses using ectromelia virus

The detailed description of the method is given in Chapter 4. Responder to stimulator ratio was 4:1. The infected stimulator cells were treated with Mitomycin-C. Cells were suspended in F-15 containing 25 µg/ml Mitomycin-C (SIGMA cat. no. M-0503) at a concentration of  $2.5 \times 10^7$  cells/ml, incubated for 30 minutes at 37°C and washed four times before responder cells were added.

#### Cytotoxicity assays

The <sup>51</sup>Cr release assays were carried out as described in Chapter 3. Macrophage targets were infected with 50 PFU/cell of virulent ectromelia

virus. Unless otherwise stated the assay time was 8 hours. Standard errors of the means were  $< 2.9$  and were omitted from tables for clarity. P815 targets were treated as L929 cells and the culture conditions are given in Chapter 6. Data given represent means (of triplicate assay wells) of specific  $^{51}\text{Cr}$  release, i.e., with spontaneous release subtracted.

#### Miscellaneous

The methods for removing dead cells and for mixed lymphocyte cultures are given in Chapters 3 and 6 respectively. Significance was determined by Student's  $t$  test.

#### RESULTS

##### Cells from radiation chimeras ( $P_1 + P_2$ ) $\rightarrow$ $F_1$ can lyse allogeneic ectromelia virus-infected cells

Chimeras were produced by reconstituting irradiated (CBA/H x C57BL/6)  $F_1$  mice with a mixture of equal numbers of CBA/H and C57BL/6 foetal liver cells. Two months later the response to ectromelia virus infection was tested: chimeras and normal CBA/H and C57BL/6 mice were given  $2 \times 10^4$  PFU of attenuated ectromelia virus and their spleen cells were tested 5 days later. C57BL/6 and CBA/H virus-immune cytotoxic cells from chimeras were isolated respectively by anti-CBA/H or anti-C57BL/6 serum and complement treatment. As a control for the effectiveness of the treatments, a mixture of equal amounts of immune T cells from CBA/H and C57BL/6 was treated with the same reagents. Table 1 illustrates that both of the individual chimeras tested contained similar numbers of lymphocytes of the two haplotypes and did not seem to contain surviving  $F_1$  cells from the host. The cytotoxic activity of untreated or complement treated samples from all three groups (both chimeras and the cell mixture) on infected CBA/H target cells was comparable. After treatment of the mixture with anti-CBA/H serum and complement, cytotoxic activity against infected CBA/H target cells was



reduced to an insignificant level. In contrast, the same treatment of the two chimeric cell populations produced little impairment of activity. These data indicate that, whereas in the mixture the only  $T_C$  cells capable of lysing infected CBA/H targets were of CBA/H origin, the chimeras contained such  $T_C$  cells which were of C57BL/6 origin. This interpretation was confirmed by the results of anti-C57BL/6 serum and complement treatment which markedly reduced the cytotoxic activity of the chimeric cell populations against infected CBA/H targets, but had no significant effect on the activity of the mixture. It is of interest to note that the data suggest that in these two chimeras the majority of  $T_C$  cells which responded to infected CBA/H cells were of C57BL/6 origin, but the reasons for this are not known.

$T_C$  cell responses of  $F_1 \rightarrow P$  chimeras to ectromelia virus-infected cells *in vivo* and *in vitro*

Individual chimeras produced by injection of  $(P_1 \times P_2) F_1$  foetal liver cells into lethally irradiated  $P_1$  or  $P_2$  hosts were either infected with virus *in vivo*, or their spleen cells were stimulated *in vitro* with infected  $F_1$  macrophages. In a number of individual chimeras, a  $T_C$  cell response was generated *in vivo* against virus-infected targets of host parental type, with minor but statistically significant activity against uninfected, but not infected, targets of the other parental type; an example of this is given in Table 2 (top line). These results essentially conform with previous findings of Bevan (1977) and Zinkernagel *et al.* (1978a).

Primary responses *in vitro* against virus-infected cells are not easily achieved, even with normal spleen cells as responders (Chapter 4). Chimeric spleens were even more difficult, possibly because of inadequate accessory cells which are crucial to the *in vitro* response (Pang and

Blanden, 1976). In consequence, only about half of the individual chimeric spleens used *in vitro* gave yields of responder cells sufficient for cytotoxicity assays, and these generally yielded only enough cells for low effector:target ratios. In two of the three individuals shown in Table 2, there was, in one respect, conformity with the results *in vivo* in that cells from  $(P_1 \times P_2) F_1 \rightarrow P_1$  chimeras gave significantly more lysis of infected than uninfected  $P_1$  targets. However, a striking new phenomenon was observed: spleen cells from  $(P_1 \times P_2) F_1 \rightarrow P_1$  chimeras gave strong lysis of uninfected targets of  $P_2$  H-2 type, and cells from  $(P_1 \times P_2) F_1 \rightarrow P_2$  chimeras gave strong lysis of uninfected  $P_1$  H-2 type. Since cells specific for uninfected targets will also lyse infected targets of the same type, it is not clear from these results whether additional subsets of cytotoxic cells specific for infected  $P_2$  and  $P_1$  targets respectively were also contributing to the observed lysis of infected targets. This question could be resolved by cold target competition, provided large enough yields can be obtained from the *in vitro* responses of individual chimeric spleens.

The same chimeric spleen cells used as responders *in vitro* (results shown in Table 2) were also tested prior to culturing, i.e., immediately upon removal from the mice. They showed no significant lysis against either parental target at an effector:target ratio of 15:1.

*In vivo* and *in vitro* responses to ectromelia virus-infected cells by  $T_C$  cells of neonatally tolerant mice

Littermates from mice shown to be tolerant to allogeneic cells *in vitro* and normal CBA/H and C57BL/6 mice were immunized with  $2 \times 10^4$  PFU of attenuated ectromelia virus and their spleen cells were tested 5 days later for lysis of virus-infected syngeneic and allogeneic target cells. Table 3 shows that  $T_C$  cells from neonatally tolerant mice lysed virus-infected syngeneic cells and not virus-infected allogeneic cells. These results



agree with those obtained from vaccinia virus-infected neonatally tolerant mice (Zinkernagel *et al.*, 1977). Since the level of  $F_1$  cells in the spleens of these mice was found to be below 5% of the total (Zinkernagel *et al.*, 1977), the lack of response to virus-infected tolerated cells could have been due to lack of stimulation through virus-infected cells in these mice. Effector cells were therefore generated *in vitro*, to provide stimulation with virus-infected cells of both haplotypes. Cultures containing ectromelia virus-infected CBA/H and C57BL/6 macrophages or (CBA/H x C57BL/6)  $F_1$  spleen cells were used (Experiments 1 and 2 respectively in Table 4). (CBA/H x C57BL/6)  $F_1$  effector cells stimulated in parallel cultures were used as controls. Table 4 shows that spleen cells from tolerant mice (mice nos. 1, 2, 3, 4) generated a response to syngeneic ectromelia virus-infected cells but not to the tolerated virus-infected cells, despite availability of stimulation from the latter as shown by responses of  $F_1$  controls.

#### Specificity of unresponsiveness

To determine the specificity of unresponsiveness, spleen cells from CBA/H and C57BL/6 mice injected at birth with  $F_1$  cells were cocultured with ectromelia virus-infected  $F_1$  spleen cells (as above) or with Mitomycin-C-treated BALB/c (H-2<sup>d</sup>) spleen cells at a responder:stimulator ratio of 4:1. Both CBA/H mice appeared to be tolerant to C57BL/6; as before there was no lysis detectable of virus-infected C57BL/6 cells either (Table 5A). The results listed for C57BL/6 mice (nos. 9 and 10) indicate that these mice were not fully tolerant. (Compare responses of C57BL/6 mice in Tables 4 and 5A). However, tolerant CBA/H, partially tolerant C57BL/6 and normal CBA/H and C57BL/6 cells all generated a comparable response against third party BALB/c stimulators (Table 5B).

#### DISCUSSION

The data given in Table 1 clearly indicate that under certain

conditions  $T_C$  cells can respond to, and lyse, virus-infected allogeneic cells, as shown previously in other laboratories (Pfizenmaier *et al.*, 1976; von Boehmer and Haas, 1976; Zinkernagel, 1976). This excludes any "physiological interaction" model for which a like-like interaction between H-2 coded self-recognition structures on effector and target cells is postulated as a prerequisite for response or lysis to occur (Zinkernagel and Doherty, 1974). However, the results are compatible with either dual recognition or altered self models, in which H-2 antigen is recognized by a clonally expressed  $T_C$  cell receptor (Blanden *et al.*, 1977; Zinkernagel and Doherty, 1977).

An unexpected finding was that in the (CBA/H + C57BL/6)  $\rightarrow$   $F_1$  chimeras the predominant lysis of infected CBA/H targets appeared to be due to  $T_C$  cells of C57BL/6 origin, although both chimeras used in this experiment seemed to be evenly reconstituted with cells of both parental types. The serological test carried out on the total splenic cell population does not exclude the possibility that a higher proportion of stem cells from C57BL/6 than from CBA/H mice entered the thymus and gave rise to the observed  $T_C$  cells.

Results from  $F_1 \rightarrow P$  chimeras and from neonatally tolerant mice (Tables 2 to 4) indicate that the H-2 antigens in the environment during  $T_C$  cell ontogeny are those which are seen as self-markers by  $T_C$  cells which respond to virus-infected cells. The  $T_C$  cell receptor repertoire apparently has the potential to cover allogeneic H-2 markers (in respect to the H-2 type of the  $T_C$  cell), but this potential is only realized by  $T_C$  cells which differentiate in an environment which expresses alloantigens. Consequently even if virally or chemically modified allogeneic (non-host) stimulation is available, only responses to modified self cells can occur (where "self" is defined by the environment of  $T_C$  cell ontogeny), e.g.,



spleen cells from neonatally tolerant mice even if cultured *in vivo* with tolerated, virus-infected, allogeneic cells (shown to be effective stimulators) do not generate a detectable response against these virus-infected allogeneic cells (Zinkernagel *et al.*, 1977; 1978b).

In recent experiments Zinkernagel *et al.* (1978b) demonstrated that  $T_C$  cells originating from  $(P_1 \times P_2) F_1$  stem cells, after maturation in an irradiated, grafted  $P_1$  thymus, respond to virus-infected  $P_1$  cells, not to virus-infected  $P_2$  cells (and *vice versa* after maturation in a  $P_2$  thymus). Thus the radiation-resistant thymic epithelial cells probably exert the determining influence of the host.

There are two ways to visualize the screening or selection process exerted on progenitor  $T_C$  cells in the thymus. The favoured interpretation by Zinkernagel *et al.* (1978a) is based on a "positive selection" model as proposed by Langman (1977). In this model, progenitor  $T_C$  cells during their differentiation in the thymus must recognize H-2 antigens on the thymic epithelium as an essential step in triggering their further maturation to the class of antigen-sensitive precursors which are capable of responding to self H-2 plus foreign antigen (X). Thus  $(P_1 \times P_2) F_1$  stem cells in a  $P_1$  thymus would not give rise to the subset of  $T_C$  cells which recognizes  $P_2$  H-2 plus X, in apparent conformity with the results by Zinkernagel *et al.* (1978a) and our observations from experiments *in vivo*. An alternative "negative selection" model as proposed by Blanden and Ada (1978) has common features with earlier proposals by Jerne (1971), Nabholz and Miggiano (1977) and Janeway *et al.* (1976). It invokes specific suppression of  $T_C$  cell precursors as follows:  $F_1$  stem cells entering the thymus are first screened for anti-self reactivity through binding to H-2K and H-2D antigens of the thymic epithelium. Since in the case of  $F_1 \rightarrow P_1$  chimeras only  $P_1$  H-2 antigens are expressed, this would result in deletion or suppression of

cells with high affinity for  $P_1$  H-2 antigens (Blanden and Ada, 1978). In contrast,  $F_1$  stem cells with affinity for  $P_2$  H-2 antigens (high as well as low affinity) are allowed to differentiate and would be able to mount an anti-self  $T_C$  cell response against  $P_2$  H-2 antigens. However, the peripheral  $T_C$  cell pool in these chimeras does maintain self-tolerance (results from *in vivo* experiments in this report and Zinkernagel *et al.*, 1978a). It is therefore proposed (Blanden and Ada, 1978) that a suppressive backlash must occur either in the thymus or in secondary lymphoid tissue, or in both. This suppressive mechanism would be specific for those  $T_C$  cells possessing receptors against  $P_2$  H-2 antigens (McCullagh 1977a; 1977b). A response to virus plus  $P_2$  H-2 antigens will therefore not be seen.

*In vitro* experiments presented here reveal lysis of uninfected targets of the parental haplotype not represented in the thymus of the irradiated host. Since only one of the uninfected parental H-2 targets was strongly lysed, it seems unlikely that it can be attributed to a response to foetal calf serum in the culture medium (Forni and Green, 1976). Further experiments have to exclude trivial explanations such as low numbers of surviving host cells responding to allogeneic cells in culture. The phenomenon reported here was not apparent in Bevan's *in vitro* experiments (1977) with similar  $F_1 \rightarrow P$  chimeras. The differences between Bevan's protocol and ours include time between reconstitution and use of chimeric spleen cells in culture, strains of mice, nature of responder cells and various details concerning the treatment of stimulator cells, type of target cells and the conditions for the cytotoxicity assay.

There was minor but statistically significant activity against uninfected targets of the appropriate parental type observed after infection for 5 days *in vivo* but spleen cells taken from uninfected



chimeras and tested immediately were inactive. The lysis of uninfected targets of the parental H-2 type not represented in the thymus of the radiation chimera after 5 days in culture may therefore reflect a specific loss of self-tolerance in the  $F_1$  population. The results suggest that some suppressor mechanism was operating against the precursors *in vivo* to maintain self-tolerance, but its effectiveness was lost during the 5 days in culture. These observations are consistent with a negative selection model, but more explicit investigation is required to determine the basis of this phenomenon.

The evidence from neonatally tolerant mice suggests that a similar suppressive mechanism operates, and is possibly more effective since it survives transition to *in vitro* conditions. A simple deletion mechanism as the basis for neonatal tolerance seems to be unlikely, since it does not explain the failure of large numbers of normal syngeneic cells to break tolerance when transferred to tolerant recipients (Billingham *et al.*, 1963; Ramseier, 1973). Also suppressor cells have been demonstrated by Dorsch and Roser (1977) in neonatally tolerant rats. So far we have been unable to provide direct experimental evidence for suppressive mechanisms in our system. Spleen cells from neonatally tolerant mice have been examined for suppressive effects on  $F_1$  cells responding to virus-infected syngeneic cells (data not shown), but *in vivo* as well as *in vitro* experiments have been inconclusive.

#### SUMMARY

In radiation chimeras and neonatally tolerant mice lymphocyte populations of one H-2 type were rendered unresponsive to allogeneic cells in order to examine whether a cytotoxic T cell response to allogeneic ectromelia virus-infected cells can be detected. Results from  $(P_1 + P_2) \rightarrow F_1$  chimeras confirm that under certain conditions such a response can be

demonstrated. Experiments using  $T_C$  cells from  $F_1 \rightarrow P$  chimeras or from neonatally tolerant mice indicate that the H-2 antigens in the environment during  $T_C$  cell ontogeny are those which are seen as self-markers by  $T_C$  cells which respond to virus-infected cells. The  $T_C$  cell receptor repertoire apparently has the potential to cover allogeneic H-2 markers (in respect to the H-2 type of the  $T_C$  cell), but this potential is only expressed by  $T_C$  cells which differentiate in an environment which expresses alloantigens. Two concepts to visualize the selection process applied to progenitor  $T_C$  cells in the thymus are discussed.

- Blanden, R. V., Pang, P. B. and Dingle, R. B. 1977. T cell recognition of virus-infected cells. in: *Virus Infection and the Cell Surface*, Cell Surface Reviews, Vol. 2, G. Poste and A. L. Nicolson, eds., North Holland Publishing Company, Amsterdam, p. 249.
- Blanden, R. V. and eds. G. L. 1978. A dual recognition model for cytotoxic T cells based on thymic selection of precursors with low affinity for self H-2 antigens. *Scand. J. Immunol.* in press.
- von Boehmer, H., Sprent, J. and Nabholz, H. 1975. Tolerance to histocompatibility determinants in tetraparental bone marrow chimeras. *J. Exp. Med.* 141:332.
- von Boehmer, H. and Haas, W. 1976. Cytotoxic T lymphocytes recognise allogeneic tolerated TNP-conjugated cells. *Nature (London)* 261:147.
- Dorsch, S. and Rager, B. 1977. Recirculating, suppressor T cells in transplantation tolerance. *J. Exp. Med.* 145:1144.
- Brooks, C. C. 1975. Neonatally induced transplantation tolerance: in vitro evidence supporting a clonal inactivation mechanism. *Eur. J. Immunol.* 5:211.
- Forni, G. and Green, I. 1976. Heterologous sera: a target for in vitro cell-mediated cytotoxicity. *J. Immunol.* 116:1561.
- Janeway Jr., C. A., Wigzell, H. and Bink, H. 1976. Two different  $V_H$  gene products make up the T cell receptors. *Scand. J. Immunol.* 8:283.
- Jerne, N. K. 1971. The somatic generation of immune recognition. *Eur. J. Immunol.* 1:1.
- Langman, R. 1977. Cell-mediated immunity and the major histocompatibility complex. *Rev. Phys. Biochem. Phys.* 11:1.
- McCullagh, P. 1977a. Anti-parental-strain lymphocyte responsiveness of  $F_1$  hybrid-strain lymphocytes. *Scand. J. Immunol.* 8:267.



## REFERENCES

- Bevan, M. J. 1977. In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. *Nature(London)* 269:417.
- Billingham, R. E., Brent, L. and Medawar, P. B. 1953. "Actively acquired tolerance" of foreign cells. *Nature(London)* 172:603.
- Billingham, R. E. and Brent, L. 1957. A simple method for inducing tolerance of skin homografts in mice. *Transplant.Bull.* 4:67.
- Billingham, R. E., Silvers, W. K. and Wilson, D. B. 1963. Further studies on adoptive transfer of sensitivity to skin homografts. *J.Exp. Med.* 118:397.
- Blanden, R. V., Pang, T. E. and Dunlop, M. B. C. 1977. T cell recognition of virus-infected cells, in: *Virus infection and the cell surface, Cell Surface Reviews, Vol. 2*, G. Poste and G. L. Nicolson, eds., North Holland Publishing Company, Amsterdam, p. 249.
- Blanden, R. V. and Ada, G. L. 1978. A dual recognition model for cytotoxic T cells based on thymic selection of precursors with low affinity for self H-2 antigens. *Scand.J.Immunol.* in press.
- von Boehmer, H., Sprent, J. and Nabholz, M. 1975. Tolerance to histocompatibility determinants in tetraparental bone marrow chimeras. *J.Exp.Med.* 141:322.
- von Boehmer, H. and Haas, W. 1976. Cytotoxic T lymphocytes recognise allogeneic tolerated TNP-conjugated cells. *Nature(London)* 261:141.
- Dorsch, S. and Roser, B. 1977. Recirculating, suppressor T cells in transplantation tolerance. *J.Exp.Med.* 145:1144.
- Brooks, C. G. 1975. Neonatally induced transplantation tolerance: *in vitro* evidence supporting a clonal inactivation mechanism. *Eur.J.Immunol.* 5:741.
- Forni, G. and Green, I. 1976. Heterologous sera: a target for *in vitro* cell-mediated cytotoxicity. *J.Immunol.* 116:1561.
- Janeway Jr., C. A., Wigzell, H. and Binz, H. 1976. Two different V<sub>H</sub> gene products make up the T cell receptors. *Scand.J.Immunol.* 5:993.
- Jerne, N. K. 1971. The somatic generation of immune recognition. *Eur.J. Immunol.* 1:1.
- Langman, R. 1977. Cell-mediated immunity and the major histocompatibility complex. *Rev.Phys.Biochem.Pharm.* 81:1.
- McCullagh, P. 1977a. Anti-parental-strain lymphocyte responsiveness of F1 hybrid-strain lymphocytes. *Scand.J.Immunol.* 6:967.

- McCullagh, P. 1977b. Anti-parental lymphocyte reactions in neonatal F1 hybrid rats. *Scand.J.Immunol.* 6:981.
- Nabholz, M. and Miggiano, V. 1977. The biological significance for the mixed lymphocyte reaction, in: *B and T cells in Immune Recognition*, F. Looz and G. E. Roelants, eds., Wiley and Sons, Chichester, England.
- Pang, T. and Blanden, R. V. 1976. The role of adherent cells in secondary cell-mediated response *in vitro* to a natural poxvirus pathogen. *Aust.J.Exp.Biol.Med.Sci.* 54:559.
- Pfizenmaier, K., Starzinski-Powitz, A., Rodt, H., Roellinghoff, M. and Wagner, H. 1976. Virus and TNP-hapten specific T cells mediated cytotoxicity against H-2 incompatible target cells. *J.Exp.Med.* 143:999.
- Ramseier, H. 1973. Antibodies to receptors recognizing histocompatibility antigens. *Current Top. Microbiol. Immunol.* 60:31.
- Streilein, J. W. and Klein, J. 1977. Neonatal tolerance induction across regions of H-2 complex. *J.Immunol.* 119:2147.
- Tulunay, Oe., Good, R. A. and Yunis, E. J. 1975. Protection of lethally irradiated mice with allogeneic fetal liver cells: Influence of irradiation dose on immunologic reconstitution. *Proc.Natl.Acad.Sci. USA* 72:4100.
- Zinkernagel, R. M. and Doherty, P. C. 1974. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature(London)* 251:547.
- Zinkernagel, R. M. 1976. H-2 restriction of virus-specific cytotoxicity across the H-2 barrier. Separate effector T cell specificities are associated with self H-2 and with the tolerated allogeneic H-2 in chimeras. *J.Exp.Med.* 141:993.
- Zinkernagel, R. M. and Doherty, P. C. 1977. Major transplantation antigens, viruses, and specificity of surveillance T cells. *Contemp.Top. Immunobiol.* 7:179.
- Zinkernagel, R. M., Callahan, G. N., Streilein, J. W. and Klein, J. 1977. Neonatally tolerant mice fail to react against virus-infected tolerated cells. *Nature(London)* 266:837.
- Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Klein, P. A. and Klein, J. 1978a. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J.Exp.Med.* 147:882.
- Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Streilein, J. W. and Klein, J. 1978b. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help. *J.Exp.Med.* 147:897.



- a Chimeras ((CBA/H + C57BL/6) → (CBA/H x C57BL/6) produced as indicated in Materials and Methods), CBA/H and C57BL/6 mice were immunized with  $2 \times 10^4$  PFU of attenuated ectromelia virus. 5 days later their spleen cells were used as effector cells. CBA/H and C57BL/6 immune cells were mixed in equal amounts.
- b The cytotoxicity assay using antisera and complement is described in Materials and Methods.
- c Effector:target ratio was 17:1 and the assay time was 17 hours. Data given are means of triplicate assay wells with lysis of uninfected target cells (< 4%) subtracted.
- d Not significantly different from chimeric cells incubated with complement only or left untreated.

TABLE 1

ECTROMELIA VIRUS-IMMUNE CELLS FROM  $(P_1 + P_2) \rightarrow F_1$  CHIMERIC MICE CAN LYSE  
 ALLOGENEIC ECTROMELIA VIRUS-INFECTED TARGET CELLS

Cells <sup>a</sup>	Serum treatment <sup>b</sup>	% Dead cells	% <sup>51</sup> Cr release from infected CBA/H targets <sup>c</sup>
Chimera A	none	-	37.1
	complement only	15	38.5
	anti-CBA/H and complement	44	33.9 <sup>d</sup>
	anti-C57BL/6 and complement	54	19.5
Chimera B	none	-	36.4
	complement only	9	36.4
	anti-CBA/H and complement	58	30.3 <sup>d</sup>
	anti-C57BL/6 and complement	50	13.7
Mixture of CBA/H and C57BL/6 cells	none	-	26.4
	complement only	14	34.2
	anti-CBA/H and complement	49	5.8
	anti-C57BL/6 and complement	51	27.8



TABLE 2

CYTOTOXIC T CELL RESPONSES TO ECTROMELIA VIRUS-INFECTED CELLS OF  $F_1 \rightarrow P$   
CHIMERAS IN VIVO AND IN VITRO

Exp.	Site of response	Origin of responder cells	Infected stimulator cells	Effector: target ratio	% Specific lysis of mac. target cells			
					C57BL/6(H-2 <sup>b</sup> )		CBA/H(H-2 <sup>k</sup> )	
					Infected	Uninfected	Infected	Uninfected
1	<i>In vivo</i>	Chimera (CBA/H x C57BL/6)F <sub>1</sub> → C57BL/6	(CBA/H x C57BL/6)F <sub>1</sub> assumed	12:1	33.2 <sup>a</sup>	3.8	4.3	10.5 <sup>b</sup>
	<i>In vitro</i>	Chimera (CBA/H x C57BL/6)F <sub>1</sub> → C57BL/6	(CBA/H x C57BL/6)F <sub>1</sub>	4:1	33.3 <sup>a</sup>	12.8 <sup>b</sup>	81.8	83.7 <sup>b</sup>
2					C57BL/6(H-2 <sup>b</sup> )		BALB/c(H-2 <sup>d</sup> )	
					Infected	Uninfected	Infected	Uninfected
2	<i>In vitro</i>	Normal (C57BL/6 x B10.D2)F <sub>1</sub> (H-2 <sup>b</sup> x H-2 <sup>d</sup> )	(C57BL/6 x B10.D2)F <sub>1</sub>	6:1	26.4 <sup>a</sup>	6.8	19.7 <sup>a</sup>	6.3
	<i>In vitro</i>	Chimera (C57BL/6 x B10.D2)F <sub>1</sub> → C57BL/6	(C57BL/6 x B10.D2)F <sub>1</sub>	6:1	15.9 <sup>a</sup>	5.6	66.6	83.8 <sup>b</sup>
	<i>In vitro</i>	Chimera (C57BL/6 x B10.D2)F <sub>1</sub> → B10.D2	(C57BL/6 x B10.D2)F <sub>1</sub>	6:1	76.2	87.6 <sup>b</sup>	1.5	1.9

a . Significantly more lysis than uninfected control targets (P < 0.01).

b . Significantly more than spontaneous release (P < 0.01).

TABLE 3

NEONATALLY TOLERANT MICE RESPONDING TO ECTROMELIA VIRUS INFECTION *IN VIVO*<sup>a</sup>

Effector cells	% <sup>51</sup> Cr release from targets <sup>b</sup>			
	CBA/H		C57BL/6	
	Infected	Uninfected	Infected	Uninfected
CBA/H normal	44.7 <sup>c</sup>	0.0	0.0	1.9
CBA/H (tolerant to C57BL/6)	35.6 <sup>c</sup>	0.0	0.0	0.1
C57BL/6 normal	1.6	8.5	47.5 <sup>c</sup>	1.3
C57BL/6 (tolerant to CBA/H)	0.0	3.3	43.4 <sup>c</sup>	3.8

a Normal and tolerant mice were immunized with  $2 \times 10^4$  PFU of attenuated ectromelia virus. Spleen cells were tested for cytotoxicity 5 days later.

b Peritoneal macrophage targets. Effector:target ratio was 20:1.

c Significantly more lysis than on uninfected targets of the same haplotype ( $P < 0.001$ ).



- a Spleen cells were cocultured with ectromelia virus-infected macrophage stimulators from CBA/H and C57BL/6 mice (1:1 mixture) in Experiment 1 and with ectromelia virus-infected  $F_1$  spleen cells in Experiment 2.
- b Peritoneal macrophage target cells. Effector:target ratios were 10:1 (Exp. 1) and 6:1 (Exp. 2) and assays were run for 10 hours (Exp. 1) and 17 hours (Exp. 2).
- c Significantly more lysis than on uninfected targets ( $P < 0.001$ ).
- d Significantly more lysis than on uninfected targets ( $P < 0.01$ ).

TABLE 4

RESPONSE TO ECTROMELIA VIRUS-INFECTED CELLS OF SPLEEN CELLS FROM NEONATALLY TOLERANT MICE *IN VITRO*

Exp.	Responder cells <sup>a</sup>	Status	Mouse no.	% <sup>51</sup> Cr release from targets <sup>b</sup>			
				CBA/H		C57BL/6	
				Infected	Uninfected	Infected	Uninfected
1	CBA/H (injected F <sub>1</sub> )	tolerant	1	28.2 <sup>c</sup>	4.4	7.4	6.7
	C57BL/6 (injected F <sub>1</sub> )	tolerant	2	5.5	6.7	39.6 <sup>c</sup>	8.7
	(CBA/H x C57BL/6) F <sub>1</sub>			18.2 <sup>d</sup>	4.7	37.4 <sup>c</sup>	10.0
2	CBA/H (injected F <sub>1</sub> )	tolerant	3	69.4 <sup>c</sup>	24.4	15.2	13.1
	CBA/H (injected F <sub>1</sub> )	tolerant	4	52.8 <sup>d</sup>	20.1	7.8	5.7
	C57BL/6 (injected F <sub>1</sub> )	not tolerant	5	74.1	83.7	52.3 <sup>c</sup>	18.0
	C57BL/6 (injected F <sub>1</sub> )	not tolerant	6	77.6	82.8	33.6 <sup>d</sup>	15.2
	(CBA/H x C57BL/6) F <sub>1</sub>			63.0 <sup>c</sup>	23.8	54.6 <sup>c</sup>	14.0



TABLE 5

## SPECIFICITY OF UNRESPONSIVENESS OF SPLEEN CELLS FROM NEONATALLY TOLERANT MICE

A. Response to ectromelia virus-infected cells *in vitro*

Responder cells	Status	Mouse no.	% <sup>51</sup> Cr release from macrophage targets <sup>a</sup>			
			CBA/H		C57BL/6	
			Infected	Uninfected	Infected	Uninfected
CBA/H (injected F <sub>1</sub> )	tolerant	7	32.5 <sup>b</sup>	16.6	2.1	9.9
CBA/H (injected F <sub>1</sub> )	tolerant	8	25.7 <sup>b</sup>	8.8	2.4	5.5
C57BL/6 (injected F <sub>1</sub> )	not tolerant	9	27.3	37.0	68.2 <sup>b</sup>	22.7
C57BL/6 (injected F <sub>1</sub> )	not tolerant	10	23.6	33.9	80.4 <sup>b</sup>	34.6
(CBA/H x C57BL/6) F <sub>1</sub>			14.7 <sup>b</sup>	0.2	32.4 <sup>c</sup>	1.6

a Effector:target ratio was 6:1.

b Significantly more lysis than on uninfected targets ( $P < 0.001$ ).

c Significantly more lysis than on uninfected targets ( $P < 0.01$ ).

TABLE 5

## SPECIFICITY OF UNRESPONSIVENESS OF SPLEEN CELLS FROM NEONATALLY TOLERANT MICE

B. Response to BALB/c stimulators *in vitro*

Responder cells	Mouse no.	% <sup>51</sup> Cr release from P815 (H-2 <sup>d</sup> ) targets	
		Effector:target ratio	
		3:1	12:1
CBA/H normal		56.3	83.4
CBA/H (injected F <sub>1</sub> )	7	40.5	82.7
CBA/H (injected F <sub>1</sub> )	8	47.7	83.5
C57BL/6 normal		34.4	76.4
C57BL/6 (injected F <sub>1</sub> )	9	48.4	82.6
C57BL/6 (injected F <sub>1</sub> )	10	52.2	83.3



# CHAPTER 8

The phenomenon of H-2 restricted T cell response was first reported by Sankaranarayanan and Doherty (1974a) and by Shearer (1974). Their observations indicated that H-2-E and H-2-D genes impose constraints on the effector reaction of T cells apparently via their molecular products on cell surfaces (Blanchard et al., 1975; Forman, 1975; Shearer et al., 1975). Two hypotheses have been advanced to explain the findings, and the following versions are currently considered:

The 'altered self' model proposed that a self-associated antigen receptor like T cells is a self-associated antigen receptor. The antigen H-2 is an endogenous antigen which negatively regulates a self-associated antigen receptor. This antigenic pattern is an altered antigenic self. This is due to one or more of the following: a change in the antigenic determinant, resulting from allelic change in one or the other, or both, of the antigenic components involved, or as a 'junction' gene.

## CONCLUDING DISCUSSION

According to the altered self model the receptor on T cells has affinity only for the complex and not for each separate component. The alternative view is that the receptor is a self-associated antigen receptor. This view is supported by the fact that H-2-E and H-2-D are expressed on each individual mouse and possess a second one for the foreign antigen. A single set of receptors (anti-H-2 and anti-X) is essential for triggering of T cell function, whereas antigen binding to two anti-H-2 or two anti-X receptors does not cause triggering.

The findings on H-2 restricted T cell response to virus infected cells suggest that these concepts have to be judged and be summarized as follows:

1. T cell response can be induced by antigen presenting cells expressing a foreign determinant and a self determinant.

The phenomenon of H-2 restricted cytotoxic T ( $T_c$ ) cell responses was first reported by Zinkernagel and Doherty (1974a) and by Shearer (1974). Their observation indicated that *H-2K* and *H-2D* genes impose constraints on the effector reaction of  $T_c$  cells apparently *via* their molecular products on cell surfaces (Blanden *et al.*, 1975b; Forman, 1975; Shearer *et al.*, 1975). Two hypotheses have been advanced to explain the findings, and the following versions are currently considered:

The 'altered self' model proposes that a single  $T_c$  cell receptor recognizes a complex antigen which is dependent on H-2 antigens and foreign antigens X (which include haptenic, viral and minor H antigens). This antigenic pattern can be visualized either as a 'new antigenic determinant', resulting from allosteric change in one or the other, or both, of the antigenic components involved, or as a 'junction zone' (Doherty *et al.*, 1976a) formed between the contributing antigens. According to the altered self model the receptor on  $T_c$  cells has affinity only for the complex and not for each separate component. The alternative 'dual recognition' model postulates two types of receptors which are expressed on each individual  $T_c$  cell, one specific for H-2 antigens and a second one for the foreign antigen X. A paired set of receptors (anti-H-2 and anti-X) is essential in triggering  $T_c$  cell functions, whereas antigen binding to two anti-H-2 or two anti-X receptors does not cause triggering.

The findings on H-2 restricted  $T_c$  cell responses to virus-infected cells against which these two concepts have to be judged may be summarized as follows:

1.  $T_c$  cell responses can be induced by modified stimulator cells expressing a foreign determinant X and a self determinant



which is coded in either the *K* or the *D* region of the *H*-2 complex (Zinkernagel and Doherty, 1974b; Pang and Blanden, 1977).

2. The self determinant recognized by  $T_c$  cells is carried on classical *H*-2 alloantigen molecules bearing 'private' specificities that have been characterized by serological means (Chapter 5; Blanden *et al.*, 1976b; Brown and Nathenson, 1977).
3. Effector  $T_c$  cells are specific for both the foreign antigen *X* and the self *H*-2 determinant on the stimulator cell used for induction of the response. They therefore lyse only target cells expressing *X* antigens and molecules coded by the appropriate *H*-2K or *H*-2D haplotype (Doherty *et al.*, 1976b).
4. 'Cold target inhibition' experiments have shown that specific reduction of lysis of radio-labelled infected target cells is caused only by virus-infected unlabelled competitors expressing the same *H*-2 haplotype and the same viral antigen(s) as the labelled target cells. Two types of unlabelled competitors are ineffective: cells expressing only the appropriate *H*-2 molecules, and virus-infected cells of an allogeneic type (Zinkernagel and Doherty, 1975).
5. Experiments reported in Chapter 6 demonstrate that the requirements for  $T_c$  cells to adsorb onto macrophage monolayers are the same as for cold target competition, i.e., adsorption is only observed in cases where the macrophages express the same *H*-2 haplotype together with the same viral antigen(s) as

the infected, labelled target cells. This observation means that H-2 restriction applies to the binding of effector and target cells, i.e., it excludes interpretations based on post-binding lytic processes.

6. Receptors on  $T_C$  cells seem to be clonally expressed, i.e., separate subsets of  $T_C$  cells are generated against K or D molecules on modified syngeneic cells (Zinkernagel and Doherty, 1974b; Pang and Blanden, 1977).
7. H-2 restriction applies also to the expression of the antiviral effects of virus-immune T cells *in vivo* (Chapter 2). Only if donors of immune T cells share H-2K or H-2D region with the virus-infected recipient does transfer of antiviral mechanisms by spleen cells occur. This clearly indicates that the original observation of H-2 restricted lysis *in vitro* has a counterpart *in vivo*.
8. The specificity of  $T_C$  cells for self H-2K in connection with viral antigens as tested *in vitro* is exquisite. For example, mutant and wild-type H-2K molecules which are qualitatively indistinguishable by serological criteria (Klein *et al.*, 1974; McKenzie *et al.*, 1976) and which crossreact markedly when recognized by alloreactive third-party  $T_C$  cells (Forman and Klein, 1975; Melief *et al.*, 1975) do not crossreact when recognized by  $T_C$  cells specific for virus antigens together with self antigens (Blanden *et al.*, 1976b; Pang *et al.*, 1977).
9. Evidence from secondary *in vitro* responses to ectromelia virus-infected cells suggests that cooperation between different Ly-subclasses of T cells is required for the



generation of  $T_C$  cells *in vitro* (Pang *et al.*, 1976). The  $T_C$  cell response seems to be facilitated by a 'helper' subclass which responds to antigenic patterns containing both virus-induced and *I* region-determined components (Pang and Blanden, 1977). This idea is also supported by *in vivo* experiments of Zinkernagel *et al.* (1978b).

10. H-2 restricted responses *in vitro* and *in vivo* have been demonstrated for a large number of viruses from different groups (see Introduction).
11. Similar findings as listed here for responses to virus-infected stimulators have been reported for  $T_C$  cell responses to chemically modified cells or to cells expressing minor H differences (where it can be tested). However, it is important to note that H-2 restriction is more rigorous for viral systems than it is, for example, in the TNP system (Burakoff *et al.*, 1976).
12. H-2 restriction is not only imposed at the level of induction of  $T_C$  cells but is exerted in the thymus during T cell ontogeny as foreshadowed by Jerne (1971), Nabholz and Miggiano (1977) and Langman (1977). Results from  $(P_1 + P_2) \rightarrow F_1$  chimeras reported in Chapter 7 indicate that responder cell populations tolerant to alloantigens can generate a response to virus-infected allogeneic cells. From observations with chimeras where  $F_1$  stem cells were allowed to mature in an irradiated, grafted thymus of  $P_1$  origin (Zinkernagel *et al.*, 1978a) and from the results reported in Chapter 7 the following conclusions can be drawn. A  $T_C$  cell response to virus-infected or chemically modified allogeneic

cells is detectable provided that (1) it is not obscured by a concurrent response to unmodified allogeneic cells or possibly suppressed by anti-idiotypic  $T_C$  cells (see below), (2) sufficient modified allogeneic cells are present to stimulate a  $T_C$  cell response (Zinkernagel *et al.*, 1978b) and (3) the thymic epithelium present during T cell ontogeny displays H-2 antigens of the same type as the modified allogeneic stimulators (Bevan, 1977; Zinkernagel *et al.*, 1978a; Chapter 7).

None of the findings listed above definitively exclude one or the other proposed model for H-2 restriction. The results from adsorption studies (Chapter 6) taken at face value are consistent with an altered self model and seem to disqualify a dual recognition model, unless modifications are made to accommodate these data. However, the concept of altered self has several inherent problems; biophysical and genetic reasons make it unlikely (Langman, 1977; Zinkernagel and Doherty, 1977; Blanden and Ada, 1978). For example, how is diversity generated while simultaneously H-2 restriction is maintained? The model contains the improbable assumption that each combination of a particular self H-2 molecule with the whole range of different antigens X results in a unique antigenic pattern. It is furthermore implied that both antigens contribute equally to the specificity of the complex antigen. What mechanism ensures that none of the two contributing antigens dominates the resulting antigenic pattern?

So far, information about the molecular arrangement of H-2 antigens and viral antigens on the surface of infected cell membranes is scarce. Experiments carried out with Sendai virus (Schrader and Edelman, 1977) suggest that H-2 antigens become physically associated



with viral antigens in infected cell membranes, but the way this happens is not clear. Results presented in Chapter 5 indicate that H-2D<sup>d</sup> dependent, ectromelia virus-immune T<sub>C</sub> cells recognize an antigenic pattern consisting of virus- and H-2D<sup>d</sup> region-coded determinants. A second H-2D<sup>d</sup> region-coded molecule H-2L (previously called D') was required for efficient stimulation of precursor T<sub>C</sub> cells, possibly because it promotes appropriate physical association of viral and H-2D<sup>d</sup> molecules. The two H-2D<sup>d</sup> region-coded antigen molecules H-2D and H-2L have a similar molecular weight of approximately 45000 daltons (Hansen *et al.*, 1977b) and H-2L is apparently recognized as a major alloantigen in its own right (Blanden and Kees, 1978; Hansen and Levy, 1978). The further analysis of T<sub>C</sub> cell responses to modified syngeneic cells expressing H-2D and H-2L molecules revealed that T<sub>C</sub> cells recognize determinants on the H-2D molecule together with associated non-self antigens X. They do not recognize H-2L in detectable numbers (Blanden and Kees, 1978). These results were obtained with two different viruses (ectromelia and Sendai virus) and with minor H antigens acting as X antigens. Therefore it is proposed that recognition of self H-2D<sup>d</sup> region-coded antigens by H-2 restricted T<sub>C</sub> cells involve antigenic determinants on the H-2D molecule, not on the H-2L molecule. In view of the current models for T<sub>C</sub> cell ontogeny, this finding poses new questions (see below).

The biochemical analysis of H-2 molecules and receptor structures on T<sub>C</sub> cells should finally provide firm evidence which will disqualify either the altered self or the dual recognition model. However, the consistently observed dual specificity of T<sub>C</sub> cell recognition and the inherent problems with the altered self concept (see above) make the dual recognition model seem more probable. Results from neonatally tolerant

mice and from radiation chimeras (Chapter 7 and Zinkernagel *et al.*, 1978a) impose further constraints on a one-receptor model. The observations can be more easily interpreted in terms of dual recognition, i.e., the  $T_C$  cell receptor for self H-2 molecules is selected for and expressed independently of the receptor for antigen X. In any concept invoking two receptors, the sequential acquisition of receptors can be postulated (Blanden and Ada, 1978) which provides a means to conceptualize the maintenance of self-tolerance. Results from chimeras (listed under point 12) strongly suggest that the screening process to eliminate anti-self activity occurs in the thymus. These findings seem to indicate that stem cells apparently have the potential to recognize as 'self' any one of the complete set of MHC antigens of the species, and it depends on the allo-antigens displayed on the thymic epithelium as to which antigen is recognized as 'self' by mature  $T_C$  cells. As a consequence, descendent  $T_C$  cells from stem cells which matured in an allogeneic thymus have the capacity to respond to modified allogeneic cells upon appropriate stimulation. Thus, the self H-2 marker expressed on  $T_C$  cells is not restricting the range of antigens which can be recognized as 'self', but the restriction is the result of a selection process which occurs in the thymus.

It is not yet understood how this selection or screening process is imposed upon progenitor T cells. The two proposed theories are based on a 'positive selection' model (Zinkernagel *et al.*, 1978a; Langman, 1977) or on a 'negative selection' model (Blanden and Ada, 1978).

According to the positive selection model, progenitor  $T_C$  cells during their differentiation in the thymus must recognize H-2 antigens on the thymic epithelium as an essential step in triggering their further maturation to the class of antigen-sensitive precursors which are capable



of responding to self H-2 plus foreign antigen X. Thus  $(P_1 \times P_2)F_1$  stem cells in a  $P_1$  thymus would not give rise to the subset of  $T_C$  cells which recognizes  $P_2$  H-2 plus X, in apparent conformity with the results by Zinkernagel *et al.* (1978a) and our observations from experiments *in vivo* (Chapter 7). The alternative negative selection model as proposed by Blanden and Ada (1978) has common features with earlier proposals by Jerne (1971), Nabholz and Miggiano (1977) and Janeway *et al.* (1976a): Stem cells entering the thymus are first screened for anti-self reactivity through binding to H-2K and H-2D antigens on the thymic epithelium. Cells expressing receptors with affinity for self H-2 determinants below a certain very low threshold will be permitted, whereas cells expressing receptors with affinity for self determinants above this threshold will be activated, threaten an anti-self attack, and, to maintain self-tolerance must subsequently either change their receptor affinity/specificity or suffer suppression or deletion. It is postulated that cells passing this first test then express their second receptor (anti-X). To maintain self-tolerance, it is also postulated that cells displaying two receptor types with anti-self specificity are deleted or suppressed (i.e., if the anti-X receptor has anti-self specificity). Since in the case of  $F_1 \rightarrow P_1$  chimeras only  $P_1$  H-2 antigens are expressed in the thymus, this would result in deletion or suppression of cells with high affinity for  $P_1$  H-2 antigens (Blanden and Ada, 1978). In contrast,  $F_1$  stem cells with affinity for  $P_2$  H-2 antigens (high as well as low affinity) are allowed to differentiate and would be able to mount an anti-self  $T_C$  cell response against  $P_2$  H-2 antigens. However, the peripheral  $T_C$  cell pool in these chimeras does maintain self-tolerance (results from *in vivo* experiments reported in Chapter 7 and Zinkernagel *et al.*, 1978a). It is therefore proposed (Blanden and Ada, 1978) that a suppressive backlash must occur either in

the thymus or in secondary lymphoid tissue, or in both. This suppressive mechanism would be specific for those  $T_C$  cells possessing receptors against  $P_2$  H-2 antigens (McCullagh, 1977a; 1977b). A response to virus plus  $P_2$  H-2 antigens will therefore not be seen. Results presented in Chapter 7 provide indirect evidence consistent with a negative selection model.

Investigations with BALB/c-H-2<sup>db</sup> mutants (Blanden and Kees, 1978) revealed that  $T_C$  cells recognize non-self antigens X together with determinants on the H-2D molecule and not with determinants on the H-2L molecule, although H-2L is a self-molecule too. Why aren't  $T_C$  cells reactive to self H-2L plus X selected? One trivial explanation might be that H-2L is not expressed on the thymic epithelial cell surface membranes. This is testable using available antisera. Alternatively, the screening process in the thymus may be more complex, and may permit only certain categories of self H-2 recognition. Further investigation of factors controlling T cell differentiation in the thymus should resolve this question.

One of the predictions from a negative selection model is that  $T_C$  cells which matured in a thymus A should have the potential to generate a response to virus-infected allogeneic cells of type B. According to the model, the selection exerted on progenitors of  $T_C$  cells in thymus A does not impose any restrictions on  $T_C$  cells with affinity for B. A minority of this latter category might have low affinity for B which, upon acquisition of a receptor for X should have the capacity to generate a  $T_C$  cell response to modified H-2 incompatible cells (with respect to the H-2 type of the thymus). Such cells cannot easily be demonstrated because of alloreactive cells which obscure any potential response to modified allogeneic cells. Mainly, three procedures have



been adopted to avoid this problem. Our own approach (data not shown) involved stimulation of responder cells with allogeneic cells in a MLR. Alloreactive cells were then adsorbed onto monolayers as described in Chapter 6 and the nonadherent cells were restimulated *in vitro* with virus-infected cells (which represents a primary response to virus-infected cells). So far, these experiments have given inconclusive results, mainly because of the technical difficulties involved in generating virus-specific responses using MLR effector cells as responder cells, possibly because necessary accessory cells appear to adhere to the monolayers too (Pang and Blanden, 1976b).

The second technique uses effector cells from a MLR culture followed by treatment with 5-bromo-2-deoxyuridine (BUdR) and light to inactivate dividing cells. Residual cells are subsequently tested for their ability to generate  $T_c$  cells upon stimulation *in vitro* with modified allogeneic cells of the type used as stimulators in the first culture, since responder cells treated with BUdR were shown to be specifically unresponsive to these particular allogeneic cells. Using this approach, Schmitt-Verhulst and Shearer (1977) demonstrated that the generation of  $T_c$  cells against TNP-modified allogeneic cells does not occur. This result might be due to the elimination of TNP-modified stimulator cells in the second culture since Janeway *et al.* (1976b) reported that despite BUdR and light treatment and  $\gamma$ -irradiation, effector  $T_c$  cells were active against the stimulating allogeneic cells as measured in a chromium release assay. The presence of such effector  $T_c$  cells readily explains the specific loss of responsiveness in secondary MLR. This idea is also supported by the finding that irradiated  $T_c$  cells (from the peak of a MLR culture) suppressed the generation of  $T_c$  cells when added in small numbers at the initiation of MLR cultures (Janeway *et al.*, 1976b; Fitch *et al.*, 1976).

The third technique to remove alloreactive cells is a filtration of immunologically naive parent  $P_1$  lymphocytes through irradiated  $(P_1 \times P_2)F_1$  mice. Thoracic duct populations from these mice were shown to be depleted of alloreactivity against  $P_2$  cells (Sprent and von Boehmer, 1976) and 94% of the collected cells have been shown to be T cells (Sprent and von Boehmer, 1976). Such negatively selected lymphocytes were subsequently restimulated in virus-infected irradiated  $F_1$  recipients (Bennink and Doherty, 1978) or restimulated *in vitro* with TNP-modified cells (Wilson *et al.*, 1977). Findings from the influenza and vaccinia systems (Bennink and Doherty, 1978) indicate that  $T_C$  cells recognizing viral antigens on allogeneic  $P_2$  cells cannot be demonstrated. However, in more recent experiments carried out with  $F_1$  mice between different parental strains,  $T_C$  cells generated against allogeneic virus-infected cells could be detected (P. Doherty, personal communication). Similarly,  $H-2^k$  cells negatively selected to  $H-2^b$  cells mediate specific lysis of TNP-modified  $H-2^b$  target cells (Wilson *et al.*, 1977). Thus negative selection procedures reveal that such clones, reactive to modified allogeneic cells, may exist in the T cell pool of normal mice, although their frequency may be low and they may not be detectable in all strains of mice.

Finally, anti-idiotypic  $T_C$  cells or antibodies (Binz and Wigzell, 1977a) might provide a tool to differentiate between a one-receptor and a two-receptor model. Specific immune unresponsiveness in mice of strain A against a given set of histocompatibility antigens of mice from strain B can be induced by immunization with autologous, antigen-specific  $T_C$  lymphoblasts (generated in a MLR culture where A cells respond to B cells) (Binz and Wigzell, 1977b). Transfer experiments indicated that the underlying mechanism is due to autoanti-idiotypic antibodies and



autoanti-idiotypic  $T_C$  cells directed against receptor structures with specificity for B on A cells. Anti-idiotypic responses could be used to differentiate between altered self and dual recognition models in the following way. Groups of mice would be immunized with a population of syngeneic  $T_C$  cells generated against syngeneic cells infected with one of two different viruses, (V1) and (V2). By this procedure anti-idiotypic responses would be generated, recognizing receptor structures on the  $T_C$  (anti-self plus V1) or on  $T_C$  (anti-self plus V2) cells. Each of the two groups of mice would be divided into two subgroups and they would be injected with either virus V1 or virus V2 and their  $T_C$  cell response tested on infected target cells. If  $T_C$  (anti-self plus V1) cells express one receptor structure (altered self model) they would generate anti-idiotypic responses directed against receptor structures on  $T_C$  (anti-self plus V1) cells, i.e., the response to V2 virus infection (in mice immunized with  $T_C$  (anti-self plus V1)) should not be impaired in comparison to control groups not immunized with  $T_C$  (anti-self plus V1) cells and should be completely abrogated in mice immunized with  $T_C$  (anti-self plus V2) cells. However, reduced lysis of V2 infected target cells after immunization with  $T_C$  (anti-self plus V1) cells is expected if these anti-idiotypic responses are directed against two types of receptor structures on  $T_C$  cells used for immunization. One of them (the anti-self H-2 recognition structure) would be common to both  $T_C$  cell sets recognizing H-2 on syngeneic cells infected with V1 or V2, which should result in a reduced response to virus V2 in mice immunized with  $T_C$  (anti-self plus V2) and  $T_C$  (anti-self plus V1).

- Kees, U. and Blanden, R. V. 1976. A single genetic element in H-2K affects mouse T-cell antiviral function in poxvirus infection. *J. Exp. Med.* 143:459.
- Blanden, R. V., Kees, U. and Dunlop, M. B. C. 1977. In vitro primary induction of cytotoxic T cells against virus-infected syngeneic cells. *J. Immunol. Methods* 16:73.
- Zinkernagel, R. M., Athagis, A., Adler, B., Blanden, R. V., Davidson, W. E., Kees, U., Dunlop, M. B. C. and Shreffler, D. C. 1977. H-2 restriction of cell-mediated immunity to an intracellular bacterium. Effector T cells are specific for histaria antigen in association with H-2I region-coded self-markers. *J. Exp. Med.* 146:1353.
- Blanden, R. V., McKenzie, I. F. C., Kees, U., Melvold, R. W. and Kohn, R. F. 1977. Cytotoxic T cell response to echovirus virus-infected cells. Different H-2 requirements for triggering precursor T-cell induction or lysis by effector T cells defined by the H-2D<sup>b</sup> mutation. *J. Exp. Med.* 146:899.
- Kees, U. and Blanden, R. V. 1977. Protective activity of secondary effector T cells generated in vitro against echovirus virus infection in vivo. *Clin. Exp. Immunol.* 30:338.
- Blanden, R. V. and Kees, U. 1978. Cytotoxic T cell responses show some restricted specificity for 'self' than for 'non-self' H-2D-coded antigens. *J. Exp. Med.* In press.
- Kees, U., Mosilbacher, A. and Blanden, R. V. 1978. Specific adsorption of cytotoxic T cells to macrophage monolayers. In preparation.
- Kees, U. and Blanden, R. V. 1978. Cytotoxic T cell response to allogeneic echovirus virus-infected cells. In preparation.

## PUBLICATIONS



- Kees, U. and Blanden, R. V. 1976. A single genetic element in *H-2K* affects mouse T-cell antiviral function in poxvirus infection. *J.Exp.Med.* 143:450.
- Blanden, R. V., Kees, U. and Dunlop, M. B. C. 1977. *In vitro* primary induction of cytotoxic T cells against virus-infected syngeneic cells. *J.Immunol.Methods* 16:73.
- Zinkernagel, R. M., Althage, A., Adler, B., Blanden, R. V., Davidson, W. F., Kees, U., Dunlop, M. B. C. and Shreffler, D. C. 1977. *H-2* restriction of cell-mediated immunity to an intracellular bacterium. Effector T cells are specific for *Listeria* antigen in association with *H-2I* region-coded self-markers. *J.Exp.Med.* 145:1353.
- Blanden, R. V., McKenzie, I. F. C., Kees, U., Melvold, R. W. and Kohn, H. I. 1977. Cytotoxic T cell response to ectromelia virus-infected cells. Different *H-2* requirements for triggering precursor T-cell induction or lysis by effector T cells defined by the BALB/c-*H-2<sup>db</sup>* mutation. *J.Exp.Med.* 146:869.
- Kees, U. and Blanden, R. V. 1977. Protective activity of secondary effector T cells generated *in vitro* against ectromelia virus infection *in vivo*. *Clin.Exp.Immunol.* 30:338.
- Blanden, R. V. and Kees, U. 1978. Cytotoxic T cell responses show more restricted specificity for 'self' than for 'non-self' *H-2D*-coded antigens. *J.Exp.Med.* *in press*.
- Kees, U., Muellbacher, A. and Blanden, R. V. 1978. Specific adsorption of cytotoxic T cells to macrophage monolayers. *In preparation*.
- Kees, U. and Blanden, R. V. 1978. Cytotoxic T cell response to allogeneic ectromelia virus-infected cells. *In preparation*.

- Alter, B. J. and Bach, F. H. 1974. Role of T-2 lymphocyte-defined and serologically defined components in the generation of cytotoxic lymphocytes. *J. Exp. Med.* 140:1417.
- Aoki, T., Haenmerling, U., de Harven, E., Boyse, E. A. and Old, L. J. 1969. Antigenic structure of cell surfaces. An immunofluorescent study of the occurrence and topography of H-2, S and Th antigens on mouse cells. *J. Exp. Med.* 130:379.
- Bach, F. H., Gerdanue, M., Goldstein, A. L., Cohn, A. and White, A. 1971. Appearance of T cell markers in bone marrow stroma-forming cells after incubation with thymosin, a thymic hormone. *Proc. Natl. Acad. Sci. USA* 68:2734.
- Bach, F. H., Cantor, H., Scalante, G. and Sturman, G. 1975. T cell subsets: Terminology problems, in *The biological activity of thymic hormones*. D. W. van Bekkum, ed. Kooiker Scientific Publications, Rotterdam, p. 159.
- Bach, F. H. and Carnaud, C. 1976. Thymic factors. *Prog. Allergy* 21:141.
- Bailey, D. W. and Kohn, H. I. 1965. Inherited histocompatibility changes in progeny of irradiated and unirradiated mice. *Genet. Res.* 6:342.
- Bailey, D. W., Snell, G. D. and Cherry, W. 1971. Complementation and serological analysis of an H-2 variant, in *Immunogenetics of the H-2 system*, A. Longmire and J. D. Macfarlane, eds., Karger, Basel, p. 135.
- Basten, A. and Mitchell, J. 1976. Role of macrophages in T cell - B cell collaboration in antibody production, in *Immunobiology of the Macrophage*, G. S. Nelson, ed. Acad. Press, New York, p. 45.
- van Bekkum, D. W., ed. 1975. *The biological activity of thymic hormones*. Kooiker Scientific Publications, Rotterdam.
- Bennink, J. R. and O'Cherty, F. C. 1976. T cell populations specifically depleted of alloreactive potential cannot be induced to lyse H-2 different virus-infected target cells. *J. Exp. Med.* in press.
- Bevan, M. J. 1975. Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. *Nature (London)* 256:419.
- Bevan, M. J. 1977. In a radiation chimera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. *Nature (London)* 269:417.
- Binz, H. and Wigzell, H. 1977a. Antigen-binding, idiotypic T lymphocyte receptors. *Contemp. Top. Immunobiol.* 7:112.
- Binz, H. and Wigzell, H. 1977b. Induction of specific immune unresponsiveness with purified H-2 and leukocyte culture-activated T lymphoblasts as autoimmunogen. III. Proof for the existence of autoanti-idiotypic killer T cells and transfer of suppression to normal syngeneic recipients by T or B lymphocytes. *J. Exp. Med.* 147:63.

## BIBLIOGRAPHY



- Alter, B. J. and Bach, F. H. 1974. Role of H-2 lymphocyte-defined and serologically defined components in the generation of cytotoxic lymphocytes. *J.Exp.Med.* 140:1410.
- Aoki, T., Haemmerling, U., de Harven, E., Boyse, E. A. and Old, L. J. 1969. Antigenic structure of cell surfaces. An immunoferritin study of the occurrence and topography of H-2,  $\theta$  and TL alloantigens on mouse cells. *J.Exp.Med.* 130:979.
- Bach, F. H., Dardenne, M., Goldstein, A. L., Guha, A. and White, A. 1971. Appearance of T cell markers in bone marrow rosette-forming cells after incubation with thymosin, a thymic hormone. *Proc.Natl.Acad. Sci.USA* 68:2734.
- Bach, J. H., Cantor, H., Roelants, G. and Stutman, O. 1975. T cell subsets: Terminology problems, in *The biological Activity of thymic Hormones*. D. W. van Bekkum, ed. Kooyker Scientific Publications, Rotterdam, p. 159.
- Bach, F. J. and Carnaud, C. 1976. Thymic factors. *Progr.Allergy* 21:342.
- Bailey, D. W. and Kohn, H. I. 1965. Inherited histocompatibility changes in progeny of irradiated and unirradiated mice. *Genet.Res.* 6:330.
- Bailey, D. W., Snell, G. D. and Cherry, M. 1971. Complementation and serological analysis of an H-2 mutant, in *Immunogenetics of the H-2 system*, A. Lengerova and M. Vojtiskova, eds., Karger, Basel, p. 155.
- Basten, A. and Mitchell, J. 1976. Role of macrophages in T cell - B cell collaboration in antibody production, in *Immunobiology of the Macrophage*, D. S. Nelson, ed. Acad. Press, New York, p. 45.
- van Bekkum, D. W., ed. 1975. *The biological Activity of thymic Hormones*, Kooyker Scientific Publications, Rotterdam.
- Bennink, J. R. and Doherty, P. C. 1978. T cell populations specifically depleted of alloreactive potential cannot be induced to lyse H-2 different virus-infected target cells. *J.Exp.Med.* in press.
- Bevan, M. J. 1975. Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. *Nature (London)* 256:419.
- Bevan, M. J. 1977. In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. *Nature (London)* 269:417.
- Binz, H. and Wigzell, H. 1977a. Antigen-binding, idiotypic T lymphocyte receptors. *Contemp.Top.Immunobiol.* 7:113.
- Binz, H. and Wigzell, H. 1977b. Induction of specific immune unresponsiveness with purified mixed leukocyte culture-activated T lymphoblasts as autoimmunogen. III. Proof for the existence of autoanti-idiotypic killer T cells and transfer of suppression to normal syngeneic recipients by T or B lymphocytes. *J.Exp.Med.* 147:63.

- Blanden, R. V. 1970. Mechanisms of recovery from a generalized viral infection: Mousepox. I. The effects of anti-thymocyte serum. *J.Exp.Med.* 132:1035.
- Blanden, R. V. 1971a. Mechanisms of recovery from a generalized viral infection: Mousepox. II. Passive transfer of recovery mechanisms with immune lymphoid cells. *J.Exp.Med.* 133:1074.
- Blanden, R. V. 1971b. Mechanisms of recovery from a generalized viral infection: Mousepox. III. Regression of infectious foci. *J.Exp.Med.* 133:1090.
- Blanden, R. V. 1974. T cell responses to viral and bacterial infection. *Transplant.Rev.* 19:56.
- Blanden, R. V., Bower, N. A., Pang, T. E., Gardner, I. D. and Parish, C. R. 1975a. Effects of thymus-independent (B) cells and the H-2 gene complex on antiviral function of immune thymus-derived (T) cells. *Aust.J.Exp.Biol.Med.Sci.* 53:187.
- Blanden, R. V., Doherty, P. C., Dunlop, M. B. C., Gardner, I. D., Zinkernagel, R. M. and David, C. S. 1975b. Genes required for cytotoxicity against virus infected target cells in K and D regions of the H-2 complex. *Nature(London)* 254:269.
- Blanden, R. V. and Gardner, I. D. 1976. The cell-mediated immune response to ectromelia virus infection. I. Kinetics and characteristics of the primary effector T cell response *in vivo*. *Cell.Immunol.* 22:271.
- Blanden, R. V., Hapel, A. J., Doherty, P. C., and Zinkernagel, R. M. 1976a. Lymphocyte-macrophage interactions and macrophage activation in the expression of antimicrobial immunity *in vivo*, in *Immunobiology of the Macrophage*, D. S. Nelson, ed. Acad. Press, New York, p. 367.
- Blanden, R. V., Dunlop, M. B. C., Doherty, P. C., Kohn, H. I. and McKenzie, I. F. C. 1976b. Effects of four H-2K mutations on virus-induced antigens recognized by cytotoxic T cells. *Immunogenetics* 3:541.
- Blanden, R. V., Pang, T. E. and Dunlop, M. B. C. 1977a. T cell recognition of virus-infected cells, in *Virus infection and the cell surface, Cell Surface Reviews, Vol. 2*, G. Poste and G. L. Nicolson, eds., North Holland Publishing Company, Amsterdam, p. 249.
- Blanden, R. V., Kees, U., and Dunlop, M. B. C. 1977b. *In vitro* primary induction of cytotoxic T cells against virus-infected syngeneic cells. *J.Immunol.Methods* 16:73.
- Blanden, R. V. and Ada, G. L. 1978. A dual recognition model for cytotoxic T cells based on thymic selection of precursors with low affinity for self H-2 antigens. *Scand.J.Immunol.* in press.



- Blanden, R. V. and Kees, U. 1978. Cytotoxic T cell responses show more restricted specificity for 'self' than for 'non-self' H-2D-coded antigens. *J.Exp.Med.* in press.
- Blank, K. J., Freedman, H. A. and Lilly, F. 1976. T lymphocyte response to Friend virus-induced tumour cell lines in mice of strains congenic at H-2. *Nature(London)* 260:250.
- Blank, D. J. and Lilly, F. 1977. Evidence for H-2/viral protein complex on the cell surface as the basis for the H-2 restriction of cytotoxicity. *Nature(London)* 269:808.
- von Boehmer, H. and Byrd, W. J. 1972. Responsiveness of thymus cells to syngeneic and allogeneic lymphoid cells. *Nature(New Biol.)* 235:50.
- von Boehmer, H. 1973. Direct cell contact is required in the syngeneic mixed lymphocyte reaction. *Eur.J.Immunol.* 3:109.
- von Boehmer, H. and Adams, P. B. 1973. Syngeneic mixed lymphocyte reaction between thymocytes and peripheral lymphoid cells in mice: Strain specificity and nature of the target cell. *J.Immunol.* 110:376.
- Boyse, E. A., Miyazawa, M., Aoki, T. and Old, L. J. 1968. Ly-A and Ly-B. Two systems of lymphocyte-isoantigens in the mouse. *Proc.Roy.Soc.B.* 170:175.
- Braciale, T. J., Ada, G. L. and Yap, K. L. 1978. Functional and structural considerations in the recognition of virus-infected cells by cytotoxic T lymphocytes. *Contemp.Top.Mol.Immunol.* in press.
- Brown, J. L., Kato, K., Silver, J. and Nathenson, S. G. 1974. Notable diversity in peptide composition of murine H-2K and H-2D alloantigens. *Biochemistry* 13:3174.
- Brown, J. L. and Nathenson, S. G. 1977. Structural differences between parent and mutant H-2K glycoproteins from two H-2K gene mutants: B6.C-H-2<sup>ba</sup> (Hz1) and B6.H-2<sup>bd</sup> (M505). *J.Immunol.* 118:98.
- Brown, J. L., Nairn, R., and Nathenson, S. G. 1978. Structural differences between the mouse H-2D products of the mutant B10.D2.M504 (H-2<sup>da</sup>) and the parental nonmutant strain B10.D2 (H-2<sup>d</sup>). *J.Immunol.* 120:726.
- Burakoff, S. J., Germain, R. N. and Benacerraf, B. 1976. Cross-reactive lysis of trinitrophenyl (TNP)-derivatized H-2 incompatible target cells by cytolytic T lymphocytes generated against syngeneic TNP spleen cells. *J.Exp.Med.* 144:1609.
- Butchko, G. M., Armstrong, R. B., Martin, W. J. and Ennis, F. A. 1978. Influenza A viruses of the H2N2 subtype are lymphocyte mitogens. *Nature(London)* 271:66.
- Cantor, H. 1972. The effects of anti-theta antiserum upon graft-versus-host activity of spleen and lymph node cells. *Cell.Immunol.* 3:461.

- Cantor, H. and Weissman, I. 1976. Development and function of subpopulations of thymocytes and T lymphocytes. *Progr.Allergy* 20:1.
- Cantor, H. and Boyse, E. 1977. Regulation of the immune response by T cell subclasses. *Contemp.Top.Immunobiol.* 7:47.
- Cerottini, J.-C. and Brunner, K. T. 1974. Cell-mediated cytotoxicity, allograft rejection and tumor immunity. *Adv.Immunol.* 18:67.
- Ching, L. M., Marbrook, J. and Walker, K. Z. 1977. Spontaneous clones of cytotoxic T cells in culture. I. Characteristics of the response. *Cell.Immunol.* 31:284.
- Clark, S. L. 1973. The intrathymic environment. *Contemp.Top.Immunobiol.* 2:77.
- Cohen, J. J. and Patterson, C. K. 1975. Induction of theta-positive lymphocytes and lymphoblasts in mouse bone marrow by mitogens. *J.Immunol.* 114:374.
- Curman, B., Oestberg, L. and Peterson, P.A. 1978. Incorporation of murine MHC antigens into liposomes and their effect in the secondary mixed lymphocyte reaction. *Nature(London)* 272:545.
- Daniels, C. A. 1975. Mechanisms of viral neutralization, in *Viral Immunology and Immunopathology*, A. L. Notkins, ed. Acad. Press, New York, p. 79.
- David, J. R. and David, R. R. 1972. Cellular hypersensitivity and immunity. *Progr.Allergy* 16:300.
- Doherty, P.C. and Zinkernagel, R. M. 1976. Specific immune lysis of paramyxovirus-infected cells by H-2 compatible thymus-derived lymphocytes. *Immunology* 31:27.
- Doherty, P. C., Goetze, D., Trinchieri, G. and Zinkernagel, R. M. 1976a. Models for recognition of virally modified cells by immune thymus-derived lymphocytes. *Immunogenetics* 3:517.
- Doherty, P. C., Blanden, R. V. and Zinkernagel, R. M. 1976b. Specificity of virus-immune effector T cells for H-2K or H-2D compatible interactions: implications for H-antigen diversity. *Transplant. Rev.* 29:89.
- Doherty, P. C., Solter, D. and Knowles, B. B. 1977. H-2 gene expression is required for T cell-mediated lysis of virus-infected target cells. *Nature(London)* 266:361.
- Eardley, D. D., Hugenberg, J., McVay-Boudreau, L., Shen, F. W., Gershon, R. K. and Cantor, H. 1978. Immunoregulatory circuits among T cell sets. I. T-helper cells induce other T cell sets to exert feedback inhibition, *J.Exp.Med.* 147:1106.
- Engers, H. D. and MacDonald, H. R. 1976. Generation of cytolytic T lymphocytes *in vitro*. *Contemp.Top.Immunobiol.* 5:145.



- Erb, P. and Feldmann, M. 1975. The role of macrophages in the generation of T-helper cells. II. The genetic control of the macrophage-T-cell interaction for helper cell induction with soluble antigens. *J.Exp.Med.* 142:460.
- Fenner, F. 1949. Mouse-pox (infectious ectromelia of mice): a review. *J.Immunol.* 63:341.
- Fitch, F. W., Engers, H. D., Cerottini, J.-C. and Brunner, T. 1976. Generation of cytotoxic T lymphocytes *in vitro*. VII. Suppressive effect of irradiated MLC cells on CTL response. *J.Immunol.* 116:716.
- Flanagan, S. P. 1966. 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet.Res.(Camb.)* 8:295.
- Forman, J. 1975. On the role of the H-2 histocompatibility complex in determining the specificity of cytotoxic effector cells sensitized against syngeneic trinitrophenyl-modified targets. *J.Exp.Med.* 142:403.
- Forman, J. and Klein, J. 1975. Analysis of H-2 mutants. Evidence for multiple CML target specificities controlled by the H-2K<sup>b</sup> gene. *Immunogenetics* 1:469.
- Forman, J. and Vitetta, E. S. 1975. Absence of H-2 antigens capable of reacting with cytotoxic T cells on a teratoma line expressing a T/t locus antigen. *Proc.Natl.Acad.Sci.USA* 72:3661.
- Forman, J. and Klein, J. 1977. Immunogenetic analysis of H-2 mutations. II. Crossreactivity in cell-mediated lympholysis between TNP-modified cells from H-2 mutant strains. *Immunogenetics* 4:183.
- Fulginiti, V. A., Kempe, C. H., Hathaway, W. E., Pearlman, D. S., Sieber, O. F., Eller, J. J., Joyner, J. J., and Robinson, A. 1968. Progressive vaccinia in immunologically deficient individuals. *Birth Defects Orig.Art.Ser.* 4:129.
- van Furth, R., ed. 1975. *Mononuclear Phagocytes*. Blackwell Scientific Publications, Oxford.
- Gardner, I. D., Bower, N. A. and Blanden, R. V. 1974a. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. *Eur.J.Immunol.* 4:63.
- Gardner, I. D., Bower, N. A. and Blanden, R. V. 1974b. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. II. Identification of effector cells and analysis of mechanism. *Eur.J.Immunol.* 4:68.
- Gardner, I. D., Bower, N. A. and Blanden, R. V. 1975. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. III. Role of the H-2 gene complex. *Eur.J.Immunol.* 5:122.

- Gardner, I. D. and Blanden, R. V. 1976. The cell-mediated immune response to ectromelia virus infection. II. Secondary response *in vitro* and kinetics of memory T cell production *in vivo*. *Cell. Immunol.* 22:283.
- Gershon, R. K. 1974. T cell control of antibody production. *Contemp. Top. Immunobiol.* 3:1.
- Gomard, E., Duprez, V., Henin, Y. and Levy, J. P. 1976. H-2 region product as determinant in immune cytolysis of syngeneic tumour cells by anti-MSV T lymphocytes. *Nature (London)* 260:707.
- Gomard, E., Duprez, V., Reme, T., Colombani, M. J. and Levi, J. P. 1977. Exclusive involvement of H-2D<sup>b</sup> or H-2K<sup>d</sup> product in the interaction between T-killer lymphocytes and syngeneic H-2<sup>b</sup> or H-2<sup>d</sup> viral lymphomas. *J. Exp. Med.* 146:909.
- Gordon, R. D., Samelson, L. E. and Simpson, E. 1977. Selective response to H-Y antigen by F1 female mice sensitized to F1 male cells. *J. Exp. Med.* 146:606.
- Hale, A. H., Witte, O. N., Baltimore, D. and Eisen, H. N. 1978. Vesicular stomatitis virus glycoprotein is necessary for H-2 restricted lysis of infected cells by cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 75:970.
- Hansen, T. H., Cullen, S. E. and Sachs, D. H. 1977a. Immunochemical evidence for an additional H-2 region closely linked to H-2D. *J. Exp. Med.* 145:438.
- Hansen, T. H., Cullen, S. E., Melvold, R., Kohn, H., Flaherty, L. and Sachs, D. H. 1977b. Mutation in a new H-2 associated hostocompatibility gene closely linked to H-2D. *J. Exp. Med.* 145:1550.
- Hansen, T. H. and Levy, R. B. 1978. Alloantigens determined by a second D region locus elicit a strong *in vitro* cytotoxic response. *J. Immunol.* 120:1836.
- Hirsch, M. S., Nahmias, A. J., Murphy, F. A. and Kramer, J. H. 1968. Cellular immunity in vaccinia infection of mice. Anti-thymocyte serum effects on primary and secondary responsiveness. *J. Exp. Med.* 128:121.
- Holden, H. T. and Herberman, R. B. 1977. Cytotoxicity against tumour-associated antigens not H-2 restricted. *Nature (London)* 268:250.
- Hooks, J. J., Burns, W., Hayashi, K., Geis, S. and Notkins, A. L. 1976. Viral spread in the presence of neutralizing antibody: Mechanisms of persistence in foamy virus infection. *Infect. Immun.* 14:1172.
- Howe, M. L., Goldstein, A. L. and Battisto, J. R. 1970. Isogeneic lymphocyte interaction: Recognition of self antigens by cells of the neonatal thymus. *Proc. Natl. Acad. Sci. USA* 67:613.



- Humphrey, J. H. 1964. Studies on globulin and antibody production in mice thymectomized at birth. *Immunology* 7:419.
- Hurme, M., Hetherington, C. M., Chandler, P. R. and Simpson, E. 1978. Cytotoxic T cell responses to H-Y: mapping of the *Ir* genes. *J.Exp.Med.* 147:758.
- Janeway, C. A. Jr., Wigzell, H. and Binz, H. 1976a. Two different V<sup>H</sup> gene products make up the T cell receptors. *Scand.J.Immunol.* 5:993.
- Janeway, C. A. Jr., Mosier, D. E. and Tigelaar, R. E. 1976b. Specific loss of stimulator activity following mixed lymphocyte reactions is due to cytotoxic cells. *Eur.J.Immunol.* 6:747.
- Jerne, N. K. 1971. The somatic generation of immune recognition. *Eur.J.Immunol.* 1:1.
- Jung, H., Pfizenmaier, K., Starzinski-Powitz, A., Roellinghoff, M. and Wagner, H. 1978. Primary *in vitro* sensitization of virus specific cytotoxic T lymphocytes. *Immunology* 34:763.
- Katz, D. H. and Benacerraf, B. 1975. The function and interrelationship of T cell receptor, *Ir* genes and other histocompatibility gene products. *Transplant.Rev.* 22:175.
- Kees, U. and Blanden, R. V. 1976. A single genetic element in *H-2K* affects mouse T-cell antiviral function in poxvirus infection. *J.Exp.Med.* 143:450.
- Klein, J., Hauptfeld, M. and Hauptfeld, V. 1974. Serological distinction of mutants B6.C-H(21) and B.M505 from strain C57BL/6. *J.Exp.Med.* 140:1127.
- Klein, J. 1975. *Biology of the Mouse Histocompatibility-2 Complex*. Springer Verlag, Berlin.
- Klein, J., Chiang, C. L. and Wakeland, E. K. 1977. Histocompatibility antigens controlled by the *I* region of the murine *H-2* complex. III. Blocking with antisera of the *in vitro* response. *Immunogenetics* 5:445.
- Koszinowski, U. and Ertl, H. 1975. Lysis mediated by T cells and restricted by *H-2* antigens of target cells infected with vaccinia virus. *Nature(London)* 255:552.
- Lance, E. M., Cooper, S. and Boyse, E. A. 1970. Antigenic change in cell maturation in murine thymocytes. *Cell.Immunol.* 1:536.
- Langman, R. E. 1977. Cell-mediated immunity and the major histocompatibility complex. *Rev.Phys.Biochem.Pharm.* 81:1.
- Lemonnier, F., Neauport-Sautes, C., Kourilsky, F. M. and Démant, P. 1975. Relationship between private and public *H-2* specificities on the cell surface. *Immunogenetics* 2:517.

- Loor, F. 1977. The abnormal differentiation of the T-lymphoid system in the congenitally athymic (nude) mouse. *Ann.Immunol.(Paris)* 128C:719.
- Maki, T. and Howe, M. L. 1976. Primary *in vitro* sensitization of murine lymphocytes against isogeneic and allogeneic cells transformed by simian virus 40. *J.Immunol.* 117:1398.
- Marbrook, J. and Haskill, J. S. 1974. The *in vitro* response to sheep erythrocytes by mouse spleen cells: Segregation of distinct events leading to antibody formation. *Cell.Immunol.* 13:12.
- Marchalonis, J. J. 1976. Surface immunoglobulins of B and T lymphocytes: Molecular properties, association with the cell membrane and a unified model of antigen recognition. *Contemp.Top.Mol.Immunol.* 5:125.
- McCullagh, P. 1977a. Anti-parental-strain lymphocyte responsiveness of F1 hybrid-strain lymphocytes. *Scand.J.Immunol.* 6:967.
- McCullagh, P. 1977b. Anti-parental lymphocyte reactions in neonatal F1 hybrid rats. *Scand.J.Immunol.* 6:981.
- McDevitt, H. O. and Benacerraf, B. 1969. Genetic control of specific immune responses. *Adv.Immunol.* 11:31.
- McDevitt, H. O., Oldstone, M. B. A. and Pincus, T. 1974. Histocompatibility-linked genetic control of specific immune responses to viral infection. *Transplant.Rev.* 19:209.
- McKenzie, I. F. C., Morgan, G. M., Melvold, R. W. and Kohn, H. I. 1976. Serological and complementation studies in four C57BL/6 H-2 mutants. *Immunogenetics* 3:241.
- McKenzie, I. F. C., Pang, T. and Blanden, R. V. 1977. The use of H-2 mutants as models for the study of the T cell activation. *Immunol.Rev.* 35:181.
- Melief, C. J. M., Schwartz, R. S., Kohn, H. I. and Melvold, R. W. 1975. Dermal histocompatibility and *in vitro* lymphocyte reaction of three new H-2 mutants. *Immunogenetics* 2:337.
- Melvold, R. W. and Kohn, H. I. 1975. Histocompatibility gene mutation rates: H-2 and non-H-2. *Mutat.Res.* 27:415.
- Melvold, R. W. and Kohn, H. I. 1976. Eight new histocompatibility mutations associated with the H-2 complex. *Immunogenetics* 3:185.
- Metcalf, D. 1966a. The nature and regulation of lymphopoiesis of the normal and neoplastic thymus, in *The Thymus: Experimental and Clinical Studies*. A Ciba Foundation Symposium, G. E. W. Wolstenholme and R. Porter, eds. Churchill, London, p. 242.
- Metcalf, D. 1966b. *The Thymus: Recent Results in Cancer Research*, Vol. 5, Springer Verlag, Berlin.



- Metcalfe, D. and Moore, M. A. S. 1971. *Haemopoietic Cells*. North Holland Publishing Co., Amsterdam.
- Miller, J. F. A. P. 1961. Immunological function of the thymus. *Lancet* 2:748.
- Miller, J. F. A. P. and Osoba, D. 1963. Role of the thymus in the origin of immunological competence, in *Origin of immunologically competent Cells*, Ciba Found. Study Group, G. E. W. Wolstenholme and J. Knight, eds. Churchill, London, p. 62.
- Miller, J. F. A. P., Vadas, M. A., Whitelaw, A. and Gamble, J. 1975. H-2 gene complex restricts transfer of delayed-type hypersensitivity in mice. *Proc. Natl. Acad. Sci. USA* 72:5095.
- Mims, C. S. and Blanden, R. V. 1972. Antiviral action of immune lymphocytes in mice infected with lymphocytic choriomeningitis virus. *Infect. Immun.* 6:695.
- Mims, C. A. 1976. *The Pathogenesis of infectious Disease*. Acad. Press, London.
- Morgan, G. M., McKenzie, I. F. C. and Melvold, R. W. 1978. The relationship between the H-2 loss mutations of H-2<sup>da</sup> and H-2<sup>db</sup> in the mouse. *Submitted*.
- Morris, B. 1973. Effect of thymectomy on immunological responses in the sheep. *Contemp. Top. Immunobiol.* 2:39.
- Nabholz, M. and Miggiano, V. 1977. The biological significance of the mixed lymphocyte reaction, in *B and T cells in Immune Recognition*, F. Looz and G. E. Roelants, eds. Wiley and Sons, Chichester, England.
- Neauport-Sautes, C., Morello, D., Freed, J. H., Nathenson, S. G. and Démant, P. 1977. The private specificity H-2.4 and the public specificity H-2.28 of the D region are expressed on two independent polypeptide chains. *Eur. J. Immunol.* 8:511.
- Nelson, D. S., ed. 1976. *Immunobiology of the Macrophage*. Acad. Press, New York.
- Owen, J. J. T. and Raff, M. C. 1970. Studies on the differentiation of thymus derived lymphocytes. *J. Exp. Med.* 132:1216.
- Owen, J. J. T. 1972. The origins and development of lymphocyte populations, in *Ontogeny of acquired Immunity*. Ciba Foundation Symposium, Elsevier, Amsterdam, p. 35.
- Pang, T. and Blanden, R. V. 1976a. The cell-mediated immune response to ectromelia virus infection. Secondary response *in vitro*: Specificity, nature of effector and responder cells and requirements for induction of antigenic changes in stimulator cells. *Aust. J. Exp. Biol. Med. Sci.* 54:253.

- Pang, T. and Blanden, R. V. 1976b. The role of adherent cells in secondary cell-mediated response *in vitro* to a natural poxvirus pathogen. *Aust.J.Exp.Biol.Med.Sci.* 54:559.
- Pang, T., McKenzie, I. F. C. and Blanden, R. V. 1976. Cooperation between mouse T cell subpopulations in the cell-mediated response to a natural poxvirus pathogen. *Cell.Immunol.* 26:153.
- Pang, T. and Blanden, R. V. 1977. Genetic factors in the stimulation of T cell responses against ectromelia virus-infected cells: Role of H-2K, H-2D and H-2I genes. *Aust.J.Exp.Biol.Med.Sci.* 55:549.
- Pang, T., Andrew, M. E., Melvold, R. W. and Blanden, R. V. 1977. Specificity or affinity of cytotoxic T cells for self H-2K determinants apparently does not change between primary and secondary responses to ectromelia virus infection. *Aust.J.Exp.Biol.Med.Sci.* 55:39.
- Pantelouris, E. M. 1968. Absence of a thymus in a mouse mutant. *Nature (London)* 217:370.
- Parish, C. R., Chilcott, A. B. and McKenzie, I. F. C. 1976a. Low-molecular-weight Ia antigens in normal mouse serum. I. Detection and production of a xenogeneic antiserum. *Immunogenetics* 3:113.
- Parish, C. R., Chilcott, A. B. and McKenzie, I. F. C. 1976b. Low-molecular-weight Ia antigens in normal mouse serum. II. Demonstration of their T cell origin *Immunogenetics* 3:129.
- Pfizenmaier, K., Jung, H., Starzinski-Powitz, A., Roellinghoff, M. and Wagner, H. 1977. The role of T cells in anti-herpes simplex virus immunity. I. Induction of antigen-specific cytotoxic T lymphocytes. *J.Immunol.* 119:939.
- Pincus, T. and Snyder, H. W. Jr. 1975. Genetic control of resistance to viral infection in mice, in *Viral Immunology and Immunopathology*, A. L. Notkins, ed. Acad. Press, New York, p. 167.
- Pritchard, H. and Micklem, H. S. 1974. The nude (nu/nu) mouse as a model of thymus and T-lymphocyte deficiency, in *Proceedings of the first International Workshop on nude Mice*, Gustav Fischer Verlag, Stuttgart, p. 127.
- Rabinowitz, S. G. and Adler, W. H. 1973. Host defences during primary venezuelan equine encephalomyelitis virus infection in mice. I. Passive transfer of protection with immune serum and immune cells. *J.Immunol.* 110:1345.
- Raff, M. C. 1971. Surface antigenic markers for distinguishing T and B lymphocytes in mice. *Transplant.Rev.* 6:52.
- Rager-Zisman, B. and Allison, A. C. 1976. Mechanism of immunologic resistance to herpes simplex virus I (HSV I) infection. *J.Immunol.* 116:35.



- Rawls, W. E. and Tompkins, W. A. F. 1975. Destruction of virus infected cells by antibody and complement, in *Viral Immunology and Immunopathology*, A. L. Notkins, ed. Acad. Press, New York, p. 99.
- Rossen, R. D., Kasel, J. A. and Couch, R. B. 1971. The secretory immune system: its relation to respiratory viral infection. *Progr. Med. Virol.* 13:194.
- Rygaard, J. and Povlsen, C. O. 1976. The nude mouse vs. the hypothesis of immunological surveillance. *Transplant. Rev.* 28:43.
- Sachs, D. H. 1976. The Ia antigens. *Contemp. Top. Mol. Immunol.* 5:1.
- Schmitt-Verhulst, A.-M. and Shearer, G. M. 1975. Bifunctional major histocompatibility-linked genetic regulation of cell-mediated lympholysis to trinitrophenyl-modified autologous lymphocytes. *J. Exp. Med.* 142:914.
- Schmitt-Verhulst, A.-M. and Shearer, G. M. 1977. Specificity of CML and MLR clones responding to chemically modified syngeneic and allogeneic cells. *J. Supramol. Struct. Suppl.* 1:206.
- Schrader, J. W. and Edelman, G. M. 1977. Joint recognition by cytotoxic T cells of inactivated Sendai virus and products of the major histocompatibility complex. *J. Exp. Med.* 145:523.
- Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* 4:527.
- Shearer, G. M., Rehn, T. and Garbarino, C. A. 1975. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the H-2K and H-2D serological regions of the murine major histocompatibility complex. *J. Exp. Med.* 141:1348.
- Shiku, H., Kisielow, P., Bean, M. A., Takahashi, T., Boyse, E. A., Oettgen, H. F. and Old, L. J. 1975. Expression of T cell differentiation antigens on effector cells in cell-mediated cytotoxicity *in vitro*. Evidence for functional heterogeneity related to the surface phenotype of T cells. *J. Exp. Med.* 141:227.
- Shortman, K., von Boehmer, H., Lipp, J. and Hopper, K. 1975. Subpopulations of T lymphocytes. Physical separation, functional specialisation and differentiation pathways of sub-sets of thymocytes and thymus-dependent peripheral lymphocytes. *Transplant. Rev.* 25:163.
- Shreffler, D. C. and David, C. S. 1975. The H-2 major histocompatibility complex and the I immune response region: Genetic variation, function and organization. *Adv. Immunol.* 20:125.
- Silver, J. and Hood, L. 1976. Preliminary amino acid sequences of transplantation antigens: Genetic and evolutionary implications. *Contemp. Top. Mol. Immunol.* 5:35.

- Smith, G. S. and Walford, R. L. 1977. Influence of the main histocompatibility complex on ageing in mice. *Nature (London)* 270:727.
- de Sousa, M. A. B. and Parrot, D. M. V. 1969. Induction and recall in contact sensitivity in skin and draining lymph nodes of intact and thymectomized mice. *J. Exp. Med.* 130:671.
- Sprent, J. and von Boehmer, H. 1976. Helper function of T cells depleted of alloantigen-reactive lymphocytes by filtration through irradiated F1 hybrid recipients. I. Failure to collaborate with allogeneic B cells in a secondary response to sheep erythrocytes measured *in vivo*. *J. Exp. Med.* 144:617.
- Starr, S.E. and Allison, A. C. 1977. Role of T lymphocytes in recovery from murine cytomegalovirus infection. *Infect. Immun.* 17:458.
- Stutman, O. 1977. Two main features of T cell development: Thymus traffic and postthymic maturation. *Contemp. Top. Immunobiol.* 7:1.
- Tomasi, T. B. Jr. and Bienenstock, J. 1968. Secretory immunoglobulins. *Adv. Immunol.* 9:1.
- Transplantation Review* 1969, 1.
- Uetake, H. and Inada, T. 1977. Mechanism of induction of cellular immunity to virus infections: experiments with adenoviruses. *Ann. Microbiol. (Inst. Pasteur)* 128B:517.
- Wagner, H., Starzinski-Powitz, A., Roellinghoff, M., Golstein, P. and Jakob, H. 1978. T cell-mediated cytotoxic immune responses to F9 teratocarcinoma cells: Cytolytic effector T cells lyse H-2-negative F9 cells and syngeneic spermatogonia. *J. Exp. Med.* 147:251.
- Wainberg, M. A., Markson, Y., Weiss, D. W. and Doljanski, F. 1974. Cellular immunity against Rous sarcoma of chickens. Preferential reactivity against autochthonous target cells as determined by lymphocyte adherence and cytotoxicity tests *in vitro*. *Proc. Natl. Acad. Sci. USA* 71:3565.
- Warnatz, H. and Krapf, F. 1976. Studies on the specificity of *in vitro* induced lymphocytotoxicity to SV40-transformed fibroblasts. *J. Immunol.* 117:981.
- Welsh, R. M. Jr. and Zinkernagel, R. M. 1977. Heterospecific cytotoxic cell activity induced during the first three days of acute lymphocytic choriomeningitis virus infection in mice. *Nature (London)* 268:646.
- Wilson, D. B., Lindahl, K. F., Wilson, D. H. and Sprent, J. 1977. The generation of killer cells to trinitrophenyl-modified allogeneic targets by lymphocyte populations negatively selected to strong alloantigens. *J. Exp. Med.* 146:361.



- Wong, C. Y., Woodruff, J. J. and Woodruff, J. F. 1977a. Generation of cytotoxic T lymphocytes during coxsackievirus B-3 infection. I. Model and viral specificity. *J.Immunol.* 118:1159.
- Wong, C. Y., Woodruff, J. J. and Woodruff, J. F. 1977b. Generation of cytotoxic T lymphocytes during coxsackievirus B-3 infection. II. Characterization of effector cells and demonstration of cytotoxicity against viral-infected myofibers. *J.Immunol.* 118: 1165.
- Woodruff, J. F. and Woodruff, J. J. 1974. Involvement of T lymphocytes in the pathogenesis of coxsackievirus B-3 heart disease. *J.Immunol.* 113:1726.
- Woodruff, J. F. and Woodruff, J. J. 1975. T. lymphocyte interaction with viruses and virus-infected tissues. *Progr.Med.Virol.* 19:120.
- World Health Organization, 1973. Cell-mediated immunity and resistance to infection. *WHO Techn.Rep.Ser.No.519.*
- Yap, K. L. and Ada, G. L. 1977. Cytotoxic T cells specific for influenza virus-infected target cells. *Immunol.* 32:151.
- Yap, K. L. and Ada, G. L. 1978. The recovery of mice from influenza virus infection: adoptive transfer of immunity with immune T lymphocytes. *Scand.J.Immunol.* in press.
- Yap, K. L., Ada, G. L. and McKenzie, I. F. C. 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature(London)* 273:238.
- Zinkernagel, R. M. and Doherty, P. C. 1974a. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis virus with a syngeneic or semiallogeneic system. *Nature(London)* 248:701.
- Zinkernagel, R. M. and Doherty, P. C. 1974b. Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. *Nature(London)* 251:547.
- Zinkernagel, R. M. and Doherty, P. C. 1975. H-2 compatibility requirement for T cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities associated with structures coded for in H-2K or H-2D. *J.Exp.Med.* 141:1427.
- Zinkernagel, R. M. 1976. H-2 compatibility requirement for virus-specific T cell-mediated cytolysis. The H-2K structure is coded by a single cistron defined by H-2K<sup>b</sup> mutant mice. *J.Exp.Med.* 143:437.
- Zinkernagel, R. M. and Oldstone, M. B. A. 1976. Cells that express viral antigens but lack H-2 determinants are not lysed by immune thymus-derived lymphocytes but are lysed by other antiviral immune attack mechanisms. *Proc.Natl.Acad.Sci.USA* 73:3666.

- Zinkernagel, R. M. and Welsh, R. M. 1976. H-2 compatibility requirement for virus specific T cell-mediated effector functions *in vivo*. I. Specificity of T cells conferring antiviral protection against lymphocytic choriomeningitis virus is associated with H-2K and H-2D. *J.Immunol.* 117:1495.
- Zinkernagel, R. M. and Althage, A. 1977. Antiviral protection by virus-immune cytotoxic T cells: Infected target cells are lysed before infectious virus progeny is assembled. *J.Exp.Med.* 145:644.
- Zinkernagel, R. M. and Doherty, P. C. 1977. Major transplantation antigens, viruses, and specificity of surveillance T cells. *Contemp. Top.Immunobiol.* 7:179.
- Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Klein, P. A. and Klein, J. 1978a. On the thymus in the differentiation of 'H-2 self-recognition' by T cells: evidence for dual recognition? *J.Exp.Med.* 147:882.
- Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Streilein, J. W. and Klein, J. 1978b. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help. *J.Exp.Med.* 147:897.
- Zisman, B., Hirsch, M. S. and Allison, A. C. 1970. Selective effects of anti-macrophage serum, silica and anti-lymphocyte serum on pathogenesis of herpes virus infection of young adult mice. *J.Immunol.* 104:1155.
- Zisman, B., Wheelock, E. F. and Allison, A. C. 1971. Role of macrophages and antibody in resistance of mice against yellow fever virus. *J.Immunol.* 107:236.